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A CIBA FOUNDATION SYMPOSIUM

PRESERVATION AND TRANSPLANTATION OF NORMAL TISSUES

Editors for the Ciba Foundation

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PREFACE

The Ciba Foundation is an international centre, established as an educational and scientific charity under the laws of England. It owes its inception and support to its founder, Ciba Limited of Switzerland, but is administered exclusively by its distinguished Trustees.

As one part of the Foundation's activities, informal symposia or colloquia, strictly limited in membership, are arranged, to which leading research workers from different countries and different disciplines are invited. As the smallness of the group necessarily excludes many others active and interested in the subjects discussed, the proceedings are being published and made available throughout the world.

"*The Preservation and Transplantation of Normal Tissues*" represents fully the papers and discussions of a symposium which owed its inception largely to Dr. A. S. Parkes, to whom, and also to Prof P. B Medawar, the Director of the Foundation is much indebted for advice From the enthusiastic response of the members and the widespread interest taken in reports of the conference in *Nature* and *Science*, the subject appears to have been both timely and fascinating. This opportune meeting of research workers in such diverse subjects as biology, biophysics, surgery, pathology, immunology, blood transfusion, tissue culture, reproductive physiology and ophthalmology brought out much information about and many references to work in various parts of the world not readily available to those in any one country or field Apart from supplying such information it is hoped that this volume will give to all readers a sense of participation in this informal and friendly occasion.

Those who apply the research work referred to here in general or eye surgery, human endocrinology and fertility,

stockbreeding or in the administrative support of such work, will share with the research workers the need to study the wider significances of the increasing ability in human beings to replace tissues and organs and to secure the "immortality" of reproductive cells.

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M Allgöwer	Dept of Surgery, University of Basle
B Antoine	Hôpital Necker, Paris
R E Billingham	Dept of Zoology, University College, London
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R. Dale	Queen Victoria Hospital, East Grinstead
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Also representatives of the Royal Naval and Army Medical Services.

GENERAL PROBLEMS OF IMMUNITY

P. B. MEDAWAR

Tissue transplantation immunity is too large and complex a subject to be summarized even with telegraphic brevity within the time at my disposal. I shall therefore speak only of skin homografts, and more narrowly still, of skin homografts transplanted "orthotopically"—that is, into positions formerly occupied by skin. This is an expedient choice for the following reason. orthotopic skin homografts are the most exacting of all in their immunological and genetical requirements Many other tissues are easier to graft homoplastically, and many other positions are easier to graft into. "Easiness" is not a very rigorous concept, all it implies is that homografts of tumours, endocrine glands, cartilage, etc may survive under circumstances in which skin homografts manifestly do not, and conversely, that many positions into which skin might be transplanted (into the brain or cornea, for example) are much more hospitable to homografts than skin itself. A whole battery of qualifications must therefore be taken into account before what is said of skin homografts transplanted into skin can be held to apply to other tissues grafted into other positions. These qualifying clauses are all of the nature of special dispensations from the full rigours of transplantation immunity If skin homografts can be made to succeed, their hosts are most unlikely to raise immunological objections to the grafting of other tissues

A second restriction of subject matter I shall be dealing with grafts so small in proportion to the sizes of their hosts that reactions of graft against host, such as we are warned about by Dempster's recent work, may be neglected (One proof that graft-against-host reactions are indeed negligible is the acceptance by F_1 mice of grafts transplanted from

members of either of their parental inbred strains: in such a situation the graft is at least genetically qualified to react against its host, a fact which might be kept in mind in appraising Barrett's work on antigenic transformations—see below)

In spite of these self-restrictions, much must still be left unsaid. It is perhaps just excusable to leave aside the genetical theory of tissue transplantation, for this is relatively uncontroversial, and has in any case been the subject of excellent contemporary reviews (Hauschka, 1952; Snell, 1952)

Biological "Laws" of Transplantation.

The quantitative analysis of skin transplantation immunity must be founded upon some measurement of the intensity of the "homograft reaction" Such a measurement is the length of time for which the grafted tissue survives its transplantation: the shorter the time, the more violent the reaction. One can get a rough idea of the survival time of a skin homograft by merely watching the transition from sound healing to abject necrosis, but such appearances may be highly misleading. The accurate measurement of survival times has been described elsewhere (Medawar, 1944, 1945): it is exactly analogous to the computation of LD_{50}'s by the toxicologist, and the estimate it affords is of the *median survival time* (M S T.), i e. the time after grafting at which tissue breakdown is just complete in the grafts carried by 50 per cent of the experimental subjects.

By such means, and after standardization of the variables discussed below, we have found that skin homografts survive for the same lengths of time in males and in virgin or non-pregnant females, and over the age-interval from puberty to full growth and somewhat beyond The following data give some idea of the *tempo* of the reaction elicited by skin homografts.

The original sources should be consulted for details of graft dosage (roughly comparable in all the animals mentioned except cattle) and operative procedures. Clinical evidence,

Species	Survival Time (days)	Reference
Mice		
CBA to A	10 4±0 3 M S T ± Standard Error	Billingham, Brent, Medawar, and Sparrow (unpub)
A to CBA	11 0±0 8 ,, ,, ,,	
aaUU to A	9 1±0 4 ,, ,, ,,	
A to aaUU	9 0±0 8 ,, ,, ,,	
Rabbits		
Between heterogeneous rabbits chosen at random	10 4±1 1 M S.T. ± Standard Error	Medawar (1944, 1945),
Guinea-pigs		
Between heterogeneous stock	9 4±0 5 M S T. ± Standard Error	Sparrow (1953)
Chicks (2 weeks old)		
Rhode Island Red to White Leghorn	<9 days	Billingham, Brent, and Medawar (unpub)
Cattle		
Between unrelated cattle, or related cattle other than twins	⩽15 days	Anderson, Billingham, Lampkin, and Medawar (1951), Billingham, Lampkin, Medawar, and Williams (1952)

though much of it relates to stressed patients, suggests that skin homografts in man live somewhat longer than these figures would incline one to predict.

Before discussing the variables that influence the homograft reaction, it should be made clear that its onset is preceded by a latent period during which homografts are indistinguishable from autografts transplanted in surgically equivalent ways. They heal into place ("take") just as securely, they are as quickly and as richly vascularized, and, if the reaction is delayed long enough, they undergo the same cycle of reparative hyperplasia and differentiation of hairs and glands. (Scothorne and McGregor's careful work (1953) shows that doubts which have lately arisen about the adequacy of the vascularization of homografts are not well founded) The existence of a latent period shows that there is no "natural" immunity to skin homografts in the sense in which a recipient of blood group A can be said to be naturally immune to red cells transfused from a donor of group B

Other things being equal, the survival time of homografts varies inversely with the quantity of skin that is grafted the larger the amount grafted, the shorter is its expectation of life.* The dosage effect is weak, and, so far as skin is concerned, of altogether minor clinical importance, in rabbits, for example, a reduction of the initial graft dosage to one-eighth increases the median survival time by only about one half. The dosage phenomenon made it possible to show that the homograft reaction is "systemic", i e. is a visitation upon the graft of a state of intolerance which, once called into being, comes into force with equal vigour over the entire skin surface and possibly in any position in which grafts are as quickly vascularized as they are in skin. For if a minute graft is transplanted to, say, a rabbit's foot at the same

*The dosage effect is only known to obtain when grafts are transplanted in different dosages to the same lymphatic "basin" Two grafts transplanted to the territory drained by a single set of nodes have a shorter expectation of life than one, but it is not known (and may be doubted) whether they would have a shorter expectation of life when transplanted, for example, to opposite sides of the body

time as a large assembly is grafted to the chest, all the grafts break down simultaneously and with inflammatory and degenerative reactions of indistinguishable severity. Were local influences predominant, the small isolated graft should have outlived those transplanted in higher dosage elsewhere. Indeed, all skin homografts orthotopically transplanted on a single occasion from a single donor to a single recipient break down simultaneously, so that (faults of healing apart) any one graft is representative of the entire population But grafts transplanted to a single recipient from genetically diverse donors survive for very different lengths of time (Medawar, 1945, Longmire, Stone, Daniel and Goon, 1947; Dempster and Lennox, 1951). Such grafts do not behave quite independently, of course; for to whatever degree they share antigens in common they must co-operate in bringing about their downfall.

Exposure to and recovery from an "attack" of foreign skin leaves its recipient in a specially refractory or intolerant state. It is true of rabbits, mice (Billingham, Brent, and Medawar, unpub.), cattle (Billingham, Lampkin, Medawar and Williams, 1952), guinea-pigs (Sparrow, 1953) and human beings (cf. Gibson and Medawar, 1943; Longmire and Smith, 1951) that skin grafts transplanted to an animal which has already received and reacted against grafts from the same source are the victims of an accelerated homograft reaction. Blood vessels break down prematurely, reparative hyperplasia is subdued, and survival time is curtailed. I no longer believe that inhibition of mitosis (Medawar, 1946*a*) in such "second set" skin homografts is anything but a secondary consequence of vascular deprivation most of the distinctive histological appearances of second-set grafts become intelligible if we assume that the reaction between graft and immunized host first takes effect between graft and graft bed. In first-set grafts, by contrast, the battleground is *within* the graft, for it does not take effect until the graft has been thoroughly permeated by blood vessels.

It should go without saying that heightened resistance or

acquired immunity does not necessarily visit itself upon grafts from a donor other than that which was responsible for the preparatory immunization.

The mechanism of the immunological response

That intolerance of homoplastic grafts belongs to the general category of "actively acquired immunity reactions" is now no longer in doubt; it is, indeed, a classification which, in so general a form, has for some time ceased to be illuminating. What sort of reaction is it, or is it most nearly kin to? The answer must surely be that it is an immunity reaction *sui generis*, with its own distinctive combination of properties. Nevertheless, it could hardly fail to be more like some immunity reactions than others, and the following summary of its properties may help us to arrive at a finer classification.

(a) **Antibodies.** The perhaps naïve expectation that homografts would elicit the formation of cytotoxic antibodies demonstrable *in vitro* has not been fulfilled (Medawar, 1948), even in the highly sensitive test-system devised by Allgower, Blocker and Engley (1952); for example, phagocytosis by a donor's leucocytes *in vitro* is unaffected by incubation in a putatively immune serum, even when the leucocytes are outnumbered by lymphocytes from the draining lymph nodes of the immunized host (Sparrow, unpub.). If cytotoxic antibodies demonstrable *in vitro* really do exist they are remarkably reluctant to reveal themselves. However, Gorer (1942) working on leukæmic cells in mice, and now Billingham and Sparrow (1953) using dissociated epidermal cells in rabbits, have shown that serum immune bodies are present which, applied *in vitro*, prejudice the survival of the donor's cells when transplanted to susceptible hosts. Gorer (1937, 1947, 1950) has also shown that red cells in mice share antigens in common with the genetically defined "histocompatibility" antigens of certain tumours, the corresponding antibodies are particularly well revealed by hæmagglutination reactions in high protein media. Such a community of antigens has yet to be demonstrated in rabbits, guinea-pigs and man

(b) **Inflammation.** It is characteristic of the homograft reaction that tissue breakdown is accompanied by widespread inflammatory destruction not only of the foreign cells of the graft but also of the cells that have contributed to the formation of its stroma "Round cells" form so high a proportion of the invading leucocyte population as to have dominated the thoughts of most earlier students of transplantation immunity The most careful studies (Darcy, 1952) have, however, failed to reveal any causal connexion between lymphocytic infiltration and tissue breakdown; the role of the plasma cell is still *sub judice.* More recently, Rogers, Converse, Taylor and Campbell (unpub) have found evidence that the eosinophil is also a participant in the skin homograft reaction in human beings

(c) **The passive transfer of immunity.** Until very recently, the passive transfer of transplantation immunity had not been satisfactorily demonstrated in practice, mainly because, even in theory, it is demonstrable only in the following way An animal is grafted with tissue from a genetically foreign donor, and the graft is in due course destroyed. Serum, tissue or tissue extracts from this actively immunized primary host may now be transferred to a second animal isogenic with it. This second animal is now challenged with a graft from the original donor, or from an animal isogenic with the donor. If immunity—i e heightened resistance—has been transferred, then the test graft will live for a shorter time in the second host than in the first, the second host will behave as if it had itself been actively immunized by an earlier grafting. Any departure from this genetical design may vitiate the test, conformance with the design does not of course guarantee its validity, for the inadvertent transfer of surviving donor cells from the primary host might completely undermine it

With these principles and safeguards in mind, Mitchison (1953*a*, *b*) has now shown that enhanced resistance to certain tumour homografts in mice may be passively transferred to secondary hosts by lymph node cells, but not by serum or

colostrum (Presumably the draining lymph node lies on the anatomical pathway of the host's response, for Mitchison found that immunity was transferred by the draining nodes, but not by the contralateral nodes, after the implantation of his tumour grafts into only one side of the primary host).

(d) **The route of immunization.** Little is known of the efficacy of the several routes of immunization, but Medawar (1946) found that the heightened resistance against skin homografts conferred by a prior "grafting" of their recipient with leucocytes from their donor was at least eighteen times more effective (on a dosage basis) when the leucocytes were injected intradermally than when they were intravenously transfused

(e) **Response to pharmacologically active compounds.** The homograft reaction is subdued by cortisone (see below) but is not significantly affected by anti-histaminic drugs such as phenergan (Sparrow, unpub.; Woodruff and Boswell, unpub) or neo-antergan (Marconi, 1950).

This constellation of properties—the combination of violent inflammatory necrosis *in vivo* with the harmlessness of "immune" sera *in vitro,* the passive transfer of heightened resistance by cells but not by serum, the superiority of the intradermal over the intravenous route of immunization; the susceptibility of the reaction to cortisone but not to antihistamines, the participation of the lymphocyte and perhaps the eosinophil—points unmistakably to membership of that subgroup of immunological reactions of which tuberculin sensitivity (see Burnet and Fenner, 1949) and sensitization to simple chemical compounds are also members Mitchison has argued for a formal analogy between tissue transplantation immunity and sensitization to simple chemical compounds (such as the nitrogen and chlorine substitution products of benzene and its homologues) These compounds, it is widely thought, act by combining with and conferring immunological activity upon *native* tissue proteins. It may be that "histocompatibility" antigens differ from each other in an analogous kind of way, i e that the tissue ingredients

of different individuals are similar in their protein moieties and differ only in their immunologically active side-groupings.

If this general interpretation is correct, it should follow that tissue destruction is due to a "non-specific" inflammatory upheaval immediately consequent upon a *specific* antigen-antibody reaction which is in itself innocuous There is nothing paradoxical about such a situation, for it is paralleled by, for example, the Arthus reaction. The Arthus reaction is specific in the sense that tissue destruction is conditional upon the meeting of an antigen with its homologous antibody within the tissues; it is non-specific in the subordinate sense that the same type of tissue destruction, no doubt mediated by the same vascular-inflammatory mechanisms, is touched off by a great variety of different antigen-antibody unions With this type of analogy in mind, it need no longer be thought surprising that cytotoxic antibodies are not demonstrable by merely confronting donors' cells with immune sera *in vitro*

The antigenic stimulus; antigenic transformations.

Little is known of the antigenic stimulus Gorer's work on the sharing of serologically (and genetically) defined antigens between erythrocytes and tumour cells in mice has already been cited. Beyond this, there is abundant evidence of a widespread overlapping of the antigenic constitutions of the several tissues and organs of the individual body. All such evidence has taken the form of showing that the transplantation of a tissue A prejudices the survival of a tissue B when grafted from the same donor* to the same recipient on a later occasion Experiments of this sort are competent to reveal antigenic similarities, unfortunately, the differences are much more interesting and important, and their analysis awaits the detailed labelling of antigens either by genetical or serological methods, and preferably, as in Gorer's work, by both

Preconceptions founded upon the earliest work with

*Here and elsewhere, "the same donor" can always be taken to mean "the same donor or animals isogenic with it"

transplanted tumours have given rise to the impression that only living cells, or cells which maintain their integrity of organization in some ill-defined way, can confer immunity —i.e can protect resistant hosts against the transient growth of tumour homografts that normally occurs in the period of grace before the onset of actively acquired immunity. Certainly Darcy and I (unpub.) found no convincing evidence that immunity to skin homografts in rabbits could be provoked by tissue, which, of proved efficacy when alive, had been killed in a variety of different ways immediately before implantation (e g. by freezing and thawing, treatment with 0 01M formaldehyde or 1 100,000 proflavine, incubating in etherized serum, or heating to 50 0°C.) Barrett, Hensen, and Spilman (1951) found that a protection against tumour homografts which could be elicited by the cells in defibrinated blood was lost after physical treatments that would cause hæmolysis Yet a wealth of evidence (reviewed by Snell, 1952) shows that the growth of tumour homografts may be profoundly influenced by a preparatory treatment of their hosts with a variety of lyophilized tissues Some such treatments enhance the growth of tumours, others retard it. I do not feel that the time is yet ripe for an appraisal of this evidence, in spite of its potential importance, for much has yet to be learnt of the immunological specificity of the reactions This is a field of research in which one particularly needs accurate computations of survival times of homografts of normal tissues, for tumours seem to need comparatively little encouragement before they grow in genetically alien soil, and may therefore exaggerate the efficacy of treatments designed to promote their well-being

It is a matter of fundamental biological importance to determine whether somatic cells, *Paramecium*-like (Sonneborn, 1950) or otherwise, can "adapt" themselves to an alien environment by altering their antigenic constitutions in response to the prevailing immunological forces Losses of antigenic specificity in long-transplanted tumours are not uncommon, they have been thought to be mutations, but

Gorer (1948) points out that they may be due to reshufflings of the antigenic constitution, the apparent loss of one antigen being merely a temporary withdrawal in the face of competition from another. Analogous constitutional readjustments occur in bacteria in the course of training, and, as with bacteria, both adaptation and selection of mutants may well take place during the propagation of tumour clones (see Hauschka, 1952) Barrett and Deringer's work (1950, 1952) makes it almost certain that mutation and differential survival cannot be the whole story, for even a single passage through the F_1 generation of a cross between inbred strains may cause a profound and enduring change in the acceptability of a strain-specific tumour to the F_2 or backcross progeny. The whole theory of antigenic transformations should stand high on the agenda of future researches on transplantation immunity.

The prolongation of the life of homografts

It is a truism to say that the lifetime of a homograft can be prolonged by treatments which lower either the resistance of the host or the antigenic propensities of the graft. Only treatments of the former class will be considered in this paper, and of these, only the three with which my colleagues and I are most familiar (a) the action of adrenal hormones; (b) the effect of pregnancy; and (c) the effect of the pre-emptive exposure of animals to foreign cells in fœtal life.

(a) **The action of adrenal hormones.** It is of some theoretical importance that in the homograft reaction we have an immunological process that is sensitive to the action of certain adrenal cortical hormones To begin with rabbits: the survival of skin homografts exchanged between rabbits chosen for the maximal genetic dissimilarity is trebled or quadrupled by the subcutaneous administration of 10 mg. cortisone acetate per day (Billingham, Krohn and Medawar, 1951*a*; cf Morgan, 1951) Cortisone acts locally as well as systemically (Billingham, Krohn and Medawar, 1951*b*), for the mere inunction of homografts with 5 mg. cortisone acetate

crystals in watery suspension every third day at least doubles their survival time, so small a dose is barely effective when administered systemically. Its action is primarily to retard the development of immunity, for if the immune state has already been provoked by an earlier grafting, cortisone is almost powerless to hold its effects at bay.

Krohn (unpub) finds that Compound F (Hydrocortisone) is about as effective as cortisone, but that injections of deoxycorticosterone, progesterone, androgen and œstrogen do not prolong the lifetime of homografts. (That is not to say that they are without effect a curtailment of life would not have been revealed by experiments of this particular design). The power to retard the development of transplantation immunity is *therefore, not unexpectedly*, a property of those steroid hormones ("gluco-corticoids" in one scheme of classification) which have the power to depress the proliferation and general physiological activity of the cells of mesenchymal tissues, the family to which antibody-forming cells undoubtedly belong

Except under special circumstances that have yet to be fully defined, the administration of ACTH does *not* prolong the lifetime of skin homografts in rabbits (Krohn, unpub.); nor does it in chicks, though chick homografts respond to cortisone (Cannon and Longmire, 1952) In guinea-pigs (Sparrow, 1953 and unpub.) the converse situation obtains Homografts in guinea-pigs are most insensitive to the action of cortisone, for, in comparison with rabbits, about twice the absolute daily dose is needed to achieve half the effect ACTH administered in a slow-absorption medium in a dose of 12½ mg./day is, however, highly effective. Homografts in mice are sensitive to cortisone in systemic doses of 0 5 mg./day; a dose of 1 mg /day may actually curtail the life of homografts by causing their ischæmic mummification; their sensitivity to ACTH is not yet known. I know of no evidence that could support a confident inference about the effect of cortisone and ACTH on skin homografts in human beings. We still await the results of a number of careful

clinical trials of, say, the regular local application of cortisone as the free alcohol in a lipoid ointment base to a small sheet of "split-thickness" skin homograft which has been allowed three days, but not longer, to heal into place before the local administration begins. It is of crucial importance that cortisone should be applied to all the homografted skin upon the experimental subject, homografts left untreated provoke a concomitant immunization, the effects of which cortisone is unable to circumvent (Billingham *et al*, 1951*b*).

The principal importance of the work whose beginnings have just been summarized is its promise of discovering ground common to endocrinology and immunology—territories at present separated not so much by an iron curtain as by a wet blanket Immunologists should become aware of the fact that the homograft reaction, whatever its narrowly serological shortcomings, is a constantly reproducible, rapid, conspicuous, and regular reaction with the perhaps unique property of being harmless to its "victim", i e the host We therefore hope to persevere with the problem of the relationship between steroid hormonal activity and immunological response, regardless of whether or not its clinical promise is fulfilled.

(b) **The effect of pregnancy.** It has already been said that, other things being equal, homografts live for the same length of time on males and virgin or non-pregnant females Krohn, Heslop and Sparrow (unpub) find that homografts transplanted to female rabbits on about the twenty-second day of pregnancy (but not at the twelfth day, or after parturition) enjoy a 50–100 per cent prolongation of life Since the administration of cortisone prolongs the life of homografts on rabbits, this may well be a symptom of an enhanced secretion of cortisone-like steroids during pregnancy, whether from the hypertrophied maternal adrenals, the ovary, the fœtal adrenals, or (not inconceivably, though evidence for it is lacking) from the placenta. It has yet to be determined whether or not this prolongation of life is of adaptive significance Billingham *et al*, (1951*b*) suggested that the enhanced

secretion of cortisone-like steroids during pregnancy might in some degree protect the fœtus, *quâ* tissue homograft, against the theoretically ever-present danger of immunizing the mother with consequences harmful or even fatal to itself—an idea developed at length by Medawar (1953) with the analogy of Rh-immunization in mind

Sparrow and I are attempting to reproduce this result in mice, for although their short gestation period makes them rather unsuitable for the purpose, experiments on mice have the great advantage of being susceptible of exact genetical control The experiments on rabbits were carried out upon does in an immunologically "heterospecific" pregnancy, i.e. upon does pregnant by bucks genetically dissimilar to themselves The graft donors may well have shared antigens in common with those present in the fœtal tissue it is not inconceivable, therefore, that the modest prolongation of the life of homografts in pregnant rabbits was due to the absorption by fœtal tissue of some of the antibodies directed against the tissues of the grafts. Such a mechanism should be no less effective in pre-immunized does—does that on some earlier occasion had reacted against grafts from the same donor—than in does receiving homografts for the first time during pregnancy. Heslop, Krohn and Sparrow find, however, that the lifetime of homografts is *not* prolonged during the pregnancy of does that are already immune, a fact which brings the effect of pregnancy into line with that caused by the administration of cortisone, and which tells against our immunological misgivings With mice, of course, these misgivings can easily be set at rest. one may distinguish the effects of pregnancy by a male mouse isogenic with the female on the one hand, and isogenic with the graft donor on the other. A prolongation of life brought about by pregnancy as such should be no less effective in the former situation than in the latter

(c) **Exposure to foreign cells in fœtal life: "actively acquired tolerance".** Billingham, Brent and I are in the midst of an investigation of the mechanism by which adult

animals may become tolerant of foreign cells to which they have been exposed in fœtal life*.

It is well known that embryos are tolerant of foreign, even of "xenoplastic", grafts, and much of experimental embryology owes its existence to this dispensation; it is less well known that foreign cells introduced into embryos may live on into adult life, though their host would certainly have rejected them if exposure had been delayed until shortly after birth. The most recent demonstration of this general theorem is that of Weiss and Andres (1952), who inoculated chick embryos with melanocytes from chicks of a different strain; but the most interesting, from our point of view, is Owen's.

Owen (1945; and see Owen, Davis and Morgan, 1946, Stone, Stormont and Irwin, 1952) found that the majority of *dizygotic* cattle twins were born with red blood cells of dizygotic origin, each contained the red blood cells genetically appropriate to itself mixed with a proportion of those belonging by right of ancestry to its twin It has long been known that cattle twins are synchorial, and it need not therefore be doubted that the fœtuses exchanged red cell precursors, and perhaps other cells as well, in an exchange transfusion made possible by the confluence of their blood vessels The red cells were of course labelled by their antigenic properties, drawing upon the wealth of information amassed by Dr Irwin and his colleagues at the University of Wisconsin

Anomalous tolerance of foreign cells may be due either (a) to an antigenic adaptation of the grafted cells, or (b) to a modification of their host's immunological response Analogies could be cited in support of either interpretation, but Owen's own work makes the former most implausible, for the exchanged red cells retained the antigenic properties, without which they could not have been identified as such. Our own work (Anderson *et al.*, 1951, Billingham *et al*, 1952) shows that adaptation of the cells exchanged in fœtal life cannot be the explanation Such an adaptation could not possibly

*A fuller and more recent account of this work has now been published (*Nature, Lond*, 172, 603, 1953)

confer tolerance of tissues exchanged after birth, or in adult life, yet the great majority of dizygotic cattle twins, even when they are of unlike sex, will accept skin grafts exchanged between them after birth or in adult life

The process that occurs naturally in the lifetime of twin cattle has now been artificially reproduced by us in mice and chickens A single experiment will illustrate this principle The seventeen- to eighteen-day-old fœtuses of a pregnant CBA mouse were injected *in utero* with a suspension of miscellaneous tissue cells from an adult female mouse of strain A All but one of these were molested and killed after birth by the mother (a result not unrepresentative of our earlier experiments, the technique of which has now been simplified and much improved). Two months after its birth, the surviving mouse, now adult, was "challenged" with a skin graft from an A-line donor The normal expectation of life of such a graft is 11 0 days, with a standard deviation of 1 1 days This graft, however, was still alive when removed for histological examination eighty-three days later. During its residence it had grown a good pelt of albino hairs; meanwhile its host had received and thrown off a graft from a donor of an unrelated black strain (aaUU), so demonstrating the specificity of its acquired tolerance of A-line cells

With chicks we have made White Leghorn embryos the recipients and Rhode Island Reds the donors; at the tenth day of embryonic life the White Leghorns have been injected intravenously with 0 1–0 2 ml washed blood cells or have been grafted with miscellaneous tissue fragments upon their chorio-allantoic membranes, in either case from ten-day Rhode Island Red embryo donors. Two weeks after birth, the subjects of these operations were challenged with skin grafted from Rhode Island Red donors of the same age. The expectation of life of such grafts on normal subjects is well below ten days—a result conforming exactly with the experience of Cannon and Longmire (1952), whose technique of grafting we have gratefully adopted One such test-graft is still living after three months on a host which had been the subject of

a chorio-allantoic grafting; one of the recipients of transfused blood has somewhat reluctantly (see below) tolerated its test graft for more than thirty days In working with chicks we are handicapped by lack of genetic uniformity within our breeds, and our work is still in a purely exploratory stage

These results do little more at present than to establish, for tissue iso-antigens, a theorem first propounded by Burnet (see Burnet and Fenner, 1949) *if a young animal is confronted with an antigen before it is capable of responding by the formation of specific antibodies, then its capacity to do so in later life is reduced or wholly suppressed.* Whether or not this theorem is of general immunological validity has yet to be seen, Burnet, Stone and Edney (1950), in experiments on chicks embodying exactly the same principle, have been unable to confirm it for exposure to such antigens as human red cells, influenza virus, or bacterial virus C16 Perhaps the harmlessly self-reproductive activity of the cells which are the vehicles of tissue iso-antigens is a technically indispensable ingredient of our proof.

It is now clear that the response of animals to living foreign homologous cells undergoes a strange immunological inversion during the course of life shortly before birth, exposure leads to acquired tolerance, and this, as we have seen, is due to a modification of the host's response and not to adaptation of the grafts; shortly after birth, exposure leads to acquired immunity· the host becomes not more but very decidedly less tolerant of cells later grafted from the same donor. It is implicit in the results obtained by Cannon and Longmire (1952) that birth in chicks is the critical age, for a small proportion of skin grafts exchanged between newly-born chicks do survive their transplantation for many months. This has also been our experience with mice, but the evidence on this score is not yet complete Acquired tolerance, it should be said, is not an all-or-none phenomenon. With chicks and mice, as in our earlier work with cattle, there is every degree of tolerance, from that which allows a test graft

a few days of grace beyond its normal expectation of life to something amounting to complete acceptance. Even grafts which survive for many months may go through one or more "crises" of inflammation and cellular infiltration

What bearing has this "acquired tolerance" upon the clinical problems of homografts, i e. the problem of making homografts successful where at present they are not? On the face of it, very little, but we must not lower the boats prematurely Actively acquired tolerance is a property conferred upon embryos by *complete* iso-antigens, i e. by living foreign cells fully qualified, in an adult host, to elicit the full rigours of immunity. In some unknown way these antigens, flooding the still unresponsive host, forestall and specifically inhibit the development of a particular antibody-forming mechanism. This has only the form of an explanation, it is little more than a redescription of the facts, but it does suggest that if the adults were flooded, not with complete iso-antigens, but with matter endowed with immunological specificity, the adult antibody-forming system would be put awry in an essentially similar way It is possible that the prior treatment of the recipients of tumour homografts with lyophilized tissues in the experiments reviewed by Snell (1952) achieves just this purpose, all our experience suggests that such preparations do not contain complete antigens; they *may* have immunological specificity, i e they may be tissue "haptens". Some such explanation might also accommodate the highly interesting results of Felton (1949), who found that a single relatively massive dose of type-specific pneumococcal polysaccharide abolished the resistance of mice to a later inoculation by living pneumococci of the same immunological type I therefore agree with Snell that Felton's "immunological paralysis", our own "actively acquired tolerance", and the enhancement of tumour growth by prior treatment with lyophilized tissues are phenomena related by a common general explanation. It is here that we seek further purely technical information from immunologists, does the flooding of an animal with a hapten such as, for example, an azo-dye,

modify its capacity to form antibodies to the homologous complete antigen, an azo-dye protein conjugate?

However this may be, I now feel certain that the clinical homograft problem *is* soluble, workers all over the world are discovering gaps in the immunological defences that were unheard of even five years ago, and there seems no reason to doubt that, if the homograft problem continues to be the subject of systematic and careful research, the gaps can be made a great deal wider yet

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DISCUSSION

GORER There is certainly something peculiar about iso-immune reactions Certain iso-antibodies have the most devastating results *in vivo* but are difficult to detect *in vitro*. One of the most striking phenomena is a comparison of hetero-immune and iso-immune sera on tissue cultures If we inoculate rats with mouse tissues, we get high titres of hæmagglutinins against mouse red cells and a very rapid destruction of a culture of mouse spleen or other tissue If we inoculate a pure strain of mice with tissues from another strain, we get very high titres of hæmagglutinins with red cells of the donor strain but no detectable effect on cultures of their tissues

At present we attribute this to some peculiarity of the antibody. May it not be due to some peculiarity of the antigens? We know virtually nothing about the chemistry of the iso-antigens in mice In man, it appears that the Rhesus antigens are not protein since they are not destroyed by any proteolytic enzymes The antigens that distinguish man from another species such as a rabbit are protein and this may account for the results we get in tissue culture The hetero-immune antibody may produce damage directly. The iso-antibody may produce no direct damage, the destruction of the cell being brought about in the manner outlined by Medawar.

EICHWALD Prof. Medawar, could you say something about the technique of injecting the mouse embryo, whether you were certain that the

antigen got into the embryo, and how these experiments were controlled?

MEDAWAR With regard to the technique, the embryos are merely injected through the body-wall with as fine a syringe as possible, we don't claim to be very precise about it—we just describe the injections as "intra-embryonic"

As to the nature of the controls, I take it Dr Eichwald means controls against the consequences of merely messing about with an embryo or injecting cells into it One type of control is to show, for example, that a CBA mouse rendered tolerant of A-line cells is not tolerant of cells from mice of a different strain We haven't got very far with it, but our evidence so far shows that if you take skin from a different donor strain—in our case a black strain AU—then the CBA mouse which is tolerant to A cells is just as intolerant as ever before to cells from this third black strain. An alternative type of control is to inject the CBA mouse with its own cells We haven't done that yet, because our recovery rate simply isn't high enough to make that a working proposition We shall certainly do it on chicks

PARKES At what age do you inject the mouse embryos?

MEDAWAR We get a very high mortality if we do it before fifteen days, but at fifteen to nineteen days we can normally see the embryos distinctly through the body wall We can make out the eyes and head, and we try not to inject the head. There is no particular difficulty

PARKES I thought you'd be able to see the rough outline of the embryo, I wasn't aware you could aim for one particular part

MEDAWAR Mice do vary very much in that respect, sometimes you get mice with opaque body walls and sometimes they are quite transparent

BRENT: It is possible by palpation with swabs to bring the fœtuses one by one close to the body wall itself, and so one may get a very much better view of the embryo whilst it is being injected

MOLLISON It may be of some interest that you occasionally get a rather similar kind of incompatibility in red cell transfusions that is to say, the red cells are eliminated at an increased rate but no antibodies can be demonstrated This phenomenon was first encountered in 1948 whilst studying the survival *in vivo* of previously stored red cells It was observed that in a few recipients, red cells which were expected to be eliminated gradually over a period of about one hundred and twenty days were in fact eliminated in less than sixty days, no incompatibility could be demonstrated *in vitro*

Recently we have encountered the same phenomenon and have again been unable to demonstrate any incompatibility *in vitro*, even using more sensitive methods developed in the last few years, such as the indirect antiglobulin test In one recipient transfused with fresh blood from two donors, red cells from one donor survived normally whereas those from the other were eliminated at an increased rate.

Dr L E Young and his co-workers in Rochester, New York, have encountered a similar phenomenon following transfusion in dogs Thus in one case transfused red cells were eliminated at an increased

rate although the red cells of the recipient dog possessed all the known red cell antigens, no antibodies were found in the recipient to account for the red cell destruction

I wonder whether the antibodies responsible for this apparent incompatibility are perhaps very like the ones you have described for the skin

MEDAWAR I think that is an exceedingly interesting observation It illustrates the general point that reactions which are perhaps irritating to the blood transfusion expert or to the immunologist, are just the sort of things that we ought to be examining in studying transplantation immunity That Mollison's suggestion is perfectly plausible is shown by Gorer's early work, when he proved that certain tissue antigens in mice are in fact shared between tissue cells and red cells

ZUCKERMAN Prof Medawar referred to the fact that ACTH is far less effective than cortisone in the rabbit, and he remarked on the possible action of ACTH on target organs other than the adrenal While he has all our support in his attempt to make a rapprochement between endocrinology and immunology, I hope that bringing order into immunology in this way will not provoke disorder in endocrinology I agree about the possible action of ACTH on target organs other than the adrenal cortex, but I am a bit confused by the difference in the reactions of the guinea-pig and the rabbit In the guinea-pig ACTH has a much greater effect on the grafts than cortisone. Are we dealing with different endocrinological or different immunological responses?

MEDAWAR As to any possible chaos that might be introduced into endocrinology by the idea that ACTH has target organs other than the adrenal, I can only say that, in so far as any chaos has been introduced, it has been the unaided work of endocrinologists—I am thinking, for example, of the work of Prunty and Clayton on the effect of ACTH on adrenalectomized mice * The other point is whether guinea-pigs and rabbits have a different endocrinology Evidently they do I don't think there's much evidence that the homograft reaction in the guinea-pig differs from that in the rabbit I think we are dealing with a different pattern of endocrinological reactivity

ZUCKERMAN While we may agree that the effect of ACTH is not restricted to the adrenal cortex, we also have to recognize that generalizations in endocrinology as a rule apply to several species. In this particular instance we had a reversal of response between guinea-pig and rabbit

PARKES The rabbit and the guinea-pig are notoriously unlike in many ways, and I do agree with you that it is very difficult to argue from one to the other.

*Ciba Foundation Colloquia on Endocrinology, IV, 347

GENERAL SURGICAL PROBLEMS OF TISSUE TRANSPLANTATION

W P LONGMIRE, Jr , J A. CANNON
and R A. WEBER

DESPITE almost universal agreement that the results of grafting autogenous tissues are superior to the transplantation of homologous tissues, the demand for grafts of the latter type continues to increase It is the general clinical impression that autogenous grafts of all kinds are less susceptible to infection, inflammatory reaction, absorption or other physical alteration, than comparable homologous grafts However, as methods of preservation and storage of homologous tissues have advanced, these tissues have become more readily available and the reliability of results following their transplantation has greatly improved The indications for the use of certain homologous tissues have been widened

In general, the tissues most frequently preserved and transplanted have been relatively inert materials such as bone, cartilage, fascia, cornea or blood vessels The cellular elements of such grafts do not survive permanently after transplantation, but the inert substances which form a relatively large portion of these tissues provide a supporting framework for the invading cells of the host Despite their lack of continued viability, these grafts maintain their original physical characteristics to a degree sufficient to serve their clinical physiological function.

For many years the ultimate fate after transplantation of the cellular elements in these grafts was not clearly understood and in certain tissues uncertainty still remains regarding permanent cellular survival. Thus, such tissues have generally been classified as homografts although the problems involved in their transfer differ in many aspects from the problems

involved in the successful transfer of homografts of tissues such as endocrine glands or skin, whose characteristic cells must continue to grow and reproduce for the graft to be clinically effective after transfer. It has been seriously questioned whether grafts whose cells probably do not survive should be classified as homografts any more than should plastic or metallic splints or other materials placed within the body

Clinical experience has demonstrated that for many purposes inert tissues transplanted to patients from other humans give results superior to those obtained from the use of the foreign materials available today, such as plastics and metals With present methods of preparation, the human materials eventually become an integral part of the tissues of the recipient while the foreign substances are tolerated by the body but are never truly incorporated into the tissues. There does, therefore, seem ample reason to place these grafts of inert tissues transplanted from one individual to another into a separate category.

In a previous article (Longmire, 1952), it was suggested that homografts might be further classified into *homostatic* grafts, to denote inert tissues such as bone or cartilage transplanted from one individual to another, and *homovital* grafts, for grafts whose cells must continue to grow and reproduce for the graft to be effective after similar transplantation.

There is no desire to confuse the classification of grafted tissue further nor to perpetuate undesirable terms However, there is a need in the current literature for a clear differentiation of these two types of grafts rather than continuing the indiscriminate use of the term "homograft". For instance, one of the foremost questions today in regard to the preservation of tissues concerns the necessity or the desirability of preserving the viability of the cellular components of the graft. In the case of tissues classified as homostatic grafts there seems to be ample reason to question the necessity of preserving cellular viability in all grafts. However, vital cells would

appear to be essential for the survival and successful function of the homovital graft

Detailed information is available today regarding the behaviour of the six or seven tissues most commonly utilized as homostatic grafts The clinician, when he utilizes these grafts, knows with a fair degree of accuracy the percentage of success he may anticipate and the various complications he may encounter Undoubtedly, additional information will be forthcoming from various important investigations in progress today which will help to explain certain of the clinical failures and complications which at present are unaccountable. Within carefully defined limits the clinical usefulness of grafts of this type is firmly established, and investigators have moved on to seek answers to questions regarding optimum methods of preservation and storage, refinements of technique of grafting procedures, and possible wider application On the other hand, the one hundred per cent failure of homovital grafts when transplanted in humans by methods available today makes these grafts practically worthless to the clinician Our basic knowledge is probably not sufficient at the present time to provide the solution to the problem of successful homovital grafting. Many fundamental ideas must be proposed and investigated in the laboratory, far from the bedside, before routine successful homovital grafting is achieved and has any real value in clinical medicine.

Homostatic Grafts

The two principle indications for utilizing homostatic grafts rather than autogenous grafts are: (1) At times, obtaining autogenous tissue may increase the magnitude and hazards of an operative procedure to such an extent that the use of homologous tissue may be indicated as a practical substitute. For example, substituting homologous cartilage for autogenous rib cartilage to repair a minor facial defect (2) The lack of any available autogenous tissue for transplantation, as in cases of corneal grafts, extensive bone grafts or blood vessel grafts.

Bone, cartilage, fascia, cornea and blood vessels constitute the tissues most frequently used as homostatic grafts

Bone. Wilson (1951) has reported a study of 214 patients who were subjected to 278 operations in which sterile refrigerated homologous bone grafts were used Wound infection occurred in four cases (1·4 per cent) and there was loss of the graft in two (0 7 per cent). In a follow-up study 144 patients were traced. Eighty per cent of the grafts were considered successful and 11 per cent unsuccessful Fifteen patients (9 per cent) had undergone operations too recently for a determination of the result.

In this series of cases homologous bone was obtained for transplantation from various operative procedures in which healthy uninfected bone was excised. The bone was cleaned of all soft tissue, placed in a sealed jar and stored at a temperature between −10°C. to −20°C The average period of refrigeration for all bone grafts used was sixty-nine days. Several specimens were used after preservation for more than one year Length of refrigeration of the graft did not seem to affect the clinical result

The preservation of homologous bone grafts in merthiolate solution has been reported by Reynolds, Oliver and Ramsey (1951). Their grafts were stored in a 1 : 1000 aqueous merthiolate solution which was changed every two weeks The bone was washed in sterile saline solution before it was used. They report that the process of fixation and replacement is accomplished in a similar manner in autogenous and homologous bone grafts but that the rate of healing of the homologous bone graft is retarded

The behaviour of bone grafts preserved by drying from the frozen state has been studied by Kreuz, Hyatt, Turner and Bassett (1951). Their most recent method of preservation, reported by Turner (1952), consists of freezing the tissues as rapidly as possible in temperatures below −55°C. and then dehydrating them in a Stokes machine under high vacuum at −40°C. Grafts so prepared are then sealed in vacuum tubes and stored at room temperature They can be easily shipped

and are thought to be stable chemically for prolonged periods of time. The grafts are reconstituted by the addition of distilled water before use

The healing of freeze-dried grafts, fresh autogenous grafts and frozen homogenous grafts have been compared in the experimental studies of Turner (1952) Fresh autogenous bone healed at a somewhat more rapid rate than either the frozen or the freeze-dried grafts, especially in the earlier phases of healing In certain respects the freeze-dried bone progressed somewhat more rapidly than the frozen grafts in the first few post-operative weeks However, the difference in healing after ninety days seemed to be insignificant in all grafts whether fresh or preserved. They all seemed to serve as a scaffold which new bone invaded and gradually replaced

Despite the excellent results reported in detailed clinical and experimental studies of homologous bone grafts, the majority of orthopædic surgeons still hesitate to utilize these grafts except in very rare instances. Such reluctance is probably based on three factors (1) Prejudice against the use of any homologous tissue (2) The lack of any readily available preserved bone (3) The desire to utilize the best graft available Of these three reasons, the latter is of the greatest importance

With any particular patient requiring a graft, the surgeon is always faced with a number of uncontrollable factors which may result in failure of the procedure. He is, therefore, naturally reticent to disregard one factor he may control, namely, selection of the best available graft Faced with a particular patient, who has already been hospitalized for weeks with an ununited fracture of the leg, the surgeon hesitates to select a graft that is known to heal at a "slightly slower rate" or to be unsuccessful in a "slightly higher percentage of cases" than the best graft he can obtain

If homologous bone is to be widely used in clinical surgery, it will of course be helpful to have simplified methods of preparation, storage and transportation, but the single most influential factor affecting general clinical acceptance of the

tissue will always be its reliability in clinical procedures. Possibly greater emphasis should be placed on the study of factors which influence the delay in early healing and the causes of failure in the homologous bone graft.

Blood Vessels. As the field of vascular surgery is expanded, the demand for arterial homografts will almost certainly increase. Unlike the relatively abundant supply of autogenous bone, autogenous vascular grafts can rarely be obtained without risk of incurring a serious circulatory impairment to some part of the body

The desirability of maintaining the vitality of the graft during storage has been one of the most controversial questions involved in the investigations of arterial homografts.

Peirce, Gross, Bill and Merrell (1949) reported a close correlation between the viability of the cells of the graft when grown in tissue culture and the successful function of the graft following transplantation. Grafts placed in a balanced salt solution with 10 per cent plasma and maintained at 4°C. "took" well when transplanted and the vitality of the cells of the graft in tissue culture was high if they had not been stored longer than forty-five days. The percentage of "takes" and the percentage of viable cells in tissue culture decreased progressively after this period Peirce (1952) presents the case for the superiority of the nutrient graft as follows "It is apparent that the failures of arterial homografts of all types occur early, during that time when viably implanted tissue differs in two important ways from non-viable. (1) The lining is more physiologic and would be expected to resist thrombosis (2) Many of the cells are living and capable of participating in healing, presumably at the suture line and along the extent of the adventitia as well as in the intima This apparently endows the graft with considerable resistance to infection During this (early) period a non-viable graft appears peculiarly subject to infection and attendant disruption Possibly also, intimal changes predispose to thrombosis. A suitably preserved viable graft, on the other hand, behaves for a time much as an autograft and can actually contribute

to the healing during this critical period. In addition, it maintains a viable and relatively physiologic lining. Consequently, disruption is very infrequent and thrombosis unusual."

Coleman, Deterling and Parshley (1951) conclude on the other hand that, "viability of the aortic homograft *per se* is not a requisite for success in vascular grafts The significant difference between the percentage of demonstrable viability and subsequent functional success leaves no alternative conclusion but that viability is not the important factor it was once thought to be." These investigators have reported the successful use of frozen arterial grafts Grafts were placed in a sterile container and stored at −15°C. to −30°C.

The "freeze-drying" preservation of arterial homografts was reported by Marrangoni in 1951. This method, as previously described for the preservation of homologous bone, is at present being investigated by Pate and Sawyer at the National Naval Medical Center, Bethesda, Maryland. Preliminary reports of this work indicate a decided superiority of "freeze-dried" arterial grafts over either frozen or fresh arterial homografts The potential of the intimal surface of these grafts is said to discourage the development of thrombosis, one of the frequent causes of failure of the arterial homograft Further experimental and clinical results of the method should prove enlightening

Swann (1952) states that when viable homologous arterial grafts are transplanted, the reaction of the host destroys all the cells within the graft in three weeks The elastic tissue persists for at least two years. Within one month the graft is surrounded by firm fibrous tissue and new endothelial cells grow in from the ends of the vessel to line the graft completely.

The appearance of calcification in the intima in some of the grafts and the development of dense fibrous tissue in the adventitia suggest that observations over a period of several years will be necessary before forming a final evaluation of vascular homografts, regardless of the method of preservation. Autogenous vein grafts may prove to be superior to homolo-

gous arterial grafts for segmental replacement in arteries of moderate diameter

Cornea. Owens *et al* (1948), in reviewing the results of 417 corneal transplants, found that 36 5 per cent of the grafts remained clear The percentage of clear grafts varied greatly with the cause of the corneal opacity The best results were obtained in cases of keratoconus (65 2 per cent) and hereditary dystrophy (58 8 per cent). Poor results were obtained in cases with scars from chemical burns (20 8 per cent), trauma (18·2 per cent) or gonococcal ulcers (8 7 per cent).

The larger the size of the corneal opacity and the greater the extent of corneal vascularization, the less were the chances of obtaining a clear graft. In the group of cases with the best preoperative prognosis, about two-thirds of the grafts remained clear. In the most unfavourable cases there was only one chance in eight of the grafts remaining clear.

In regard to methods of preservation of the donor cornea, McLean (1948) states, "Postmortem eyes should be obtained as soon as possible after death, preferably within four to six hours and stored at about 4°C At present, the best method of storage is in a tightly sealed container in a saturated atmosphere of water vapour The allowable time of storage has not yet been well worked out It is best to use the donor eye as soon as possible The best index of suitability is not the elapsed time but the condition of the cornea on close inspection. In practice, it seems to make little difference whether or not the cornea be fresh or stored, as long as its condition is good at the time of operation "

Maumenee and Kornblueth (1948) studied various methods of preserving the donor corneas in transplants between rabbits. Of fifteen grafts stored in formalin, only one partially clear corneal transplant was obtained after grafting. Likewise, in a series of thirteen corneal transplants in which the cells of the graft were killed by freezing, no clear grafts were obtained For five to seven days after transplantation many of these grafts looked as promising as did fresh grafts. However, all became progressively more œdematous after the

sutures were removed and frequently the anterior lamellæ sloughed off during the first two weeks after operation

All their efforts to obtain a clear corneal graft by the use of non-viable corneas in which the cells had been killed either by freezing or by formalin were completely unsuccessful. They conclude that these experiments would indicate that living donor stromal cells, at least during the first stages of the graft, are essential for its final clarity.

Bajenova (1936) has demonstrated that corneas preserved in moist chambers at 2°C for ten days contain viable stromal cells which will grow in tissue culture. The oxygen consumption and the Q_{O_2} of stored bovine corneas have been studied by Duane (1948) He found (1) The Q_{O_2} of the cornea remains normal when stored over Ringer's solution (4°C) or under (15°C) for seven days The respiratory rate falls to one-fifth normal in ten to twelve days under these conditions (2) Formalin inhibits respiration of the cornea immediately and this is apparently irreversible (3) The corneal Q_{O_2} is reduced to one-half normal within twenty-four hours by quick freezing. Within four days the Q_{O_2} has fallen to one-fifth normal, where it remains up to sixty days storage

In regard to the ultimate fate of the corneal homograft, Maumenee and Kornblueth (1948) draw these conclusions: The majority of the stromal cells in the graft continue to live and do not show a massive degeneration or replacement They are supplemented, at least to a limited extent, by the newly formed keratoblasts from the recipient's macrophages The epithelium is replaced by a migration of epithelium from the recipient cornea The endothelium may be repaired from the uninjured cells of the graft or a migration of cells from the recipient cornea

Maumenee (1951) has obtained experimental evidence to support the theory that delayed opacification of the transplanted cornea develops as a result of an acquired immunity action. When skin from the same donor was used as an antigenic supplement, two weeks after a corneal transplantation, the corneal graft became cloudy in 28 out of 30 eyes after

insertion of the skin As transfers of the donor's skin six to eight weeks after corneal grafting into seven rabbits did not produce opacification of the cornea, it was suggested that the original donor cells of the graft had been replaced, by this time, by invading recipient cells and therefore the graft was no longer susceptible to the sensitivity reaction.

Infection, defects of the donor material, and poor apposition of the donor-recipient tissues obviously may also be responsible for clouding of corneal grafts

In summary, effective results have been obtained in 36 5 per cent of a large series of cases where cornea transplants were used in a variety of pathological conditions. The condition of the recipient cornea in regard to extent of opacification and vascularization is one of the most important factors governing the success or failure of corneal grafts in patients. Present evidence would indicate that viable grafts are superior to non-viable grafts, despite the fact that in certain cases the living cells in the graft may react to a delayed sensitivity state of the host causing opacification of the cornea A gradual replacement in the graft of all donor cells by invading host cells is probably complete within six to eight weeks In the successful graft massive simultaneous cell destruction does not occur.

Cartilage, Fascia. There is little evidence in the recent literature of any important changes in the methods of preparation, storage or utilization of homologous cartilage or fascia Homologous cartilage is most frequently stored either in a sterile container at a temperature of −10°C. to −20°C. or in a solution of 1 : 1000 aqueous merthiolate solution. No attempt is made to preserve viable cells in these tissues The incidence of infection and absorption is somewhat higher when preserved rather than autogenous cartilage is used, however, obtaining an autogenous graft may frequently convert a minor surgical procedure, that may be performed under local anæsthesia, into a major operation requiring a general anæsthetic In such cases, homologous grafts are

generally considered a practical substitute for autogenous tissue.

On the other hand, the clinical use of preserved fascia seems to have diminished. Autogenous fascia is readily obtained from the fascia lata with the fascial stripper without materially increasing the magnitude of most operative procedures and gives results superior to those obtained with preserved fascia Other autogenous tissues such as dermal grafts, full thickness skin grafts, and non-reactive metals such as tantalum are at present frequently utilized in operative procedures in preference to preserved fascia

Nerve. Davis and Ruge (1950) state that many investigators have studied the transplantation of nerve homografts, and have discarded the procedure, maintaining that the graft produces a foreign body reaction in the host and is reduced to a fibrous band or is absorbed entirely

They transplanted fresh sciatic nerve homografts in 35 cats Microscopic examination of sections of the graft indicated that the superficial layers of the graft retained their viability, whereas the deeper areas often were necrotic Where grafts were viable, the original fascicles were unchanged and the endoneural tubes remained Regeneration of the nerve was always spotty but adequate in the peripheral layers. One year following insertion of the graft all animals were capable of nearly all motions of the involved foot and toes.

Homologous nerve grafts were also performed upon ten patients The grafts were taken from recently amputated extremities or from cadavers soon after death and refrigerated for thirty minutes to forty-five hours before being used. In regard to the results they state, "while none of the patients has shown any return of motor function or enough evidence of regeneration to approach normal, some have shown minimal evidence of recovery " Three of the nerve grafts were exposed and examined four to eight months following transplantation and in no instance had the graft been absorbed or reduced to a fibrous band. In attempting to explain the markedly inferior results in man, compared to the experimental results,

the authors emphasize the importance of the condition of the graft itself In the experimental study the grafts were removed and transplanted immediately following the death of the donor. The diameter and the length of the grafts were smaller than those used in human beings. They indicate that attempts should be made to simulate the experimental conditions as closely as possible when employing nerve homografts clinically

Summary. The clinical results following the transplantation of all types of homostatic tissues leave room for improvement When using such tissues as cornea and blood vessels the clinician must accept a certain percentage of failures as he has no better alternative method available. However, autogenous grafts of bone, cartilage and fascia are utilized by the majority of surgeons despite the disadvantages of obtaining the autogenous tissue

Efforts to obtain practical methods of preserving homologous tissues, so that they may be readily transported and stored for long periods of time, should not overshadow efforts to improve the clinical results following the grafting of these tissues For example, preserved ox fascia was at one time available in many hospitals and medical supply firms in America Today this material is rarely used because the clinical results have too frequently been unsatisfactory

The importance of the vitality of the homostatic graft to its ultimate clinical success may vary from one tissue to another, depending in part upon the ratio of cellular to inert substances of the graft and also upon how closely the graft must retain its original structure to function adequately. A corneal transplant can undergo little alteration from its original state and be successful On the other hand considerable change may occur in and about the bone or cartilage transplant without destroying a final successful clinical result. In efforts to improve the clinical results of the grafting of homostatic tissue the importance of the vitality of the graft should be carefully scrutinized

Homovital Grafts

In clinical investigations, skin grafts have been studied more widely than any other type of homovital graft. Reports have also appeared concerning investigations of endocrine gland and kidney transplants

Preliminary reports of prolonged skin homograft survival following treatment of the recipient with ACTH have not been substantiated by further studies (Randall, Brown and McDowell, 1950)

It is interesting in this regard that of a number of materials tested, cortisone has been the only substance we have found to increase significantly the percentage of permanently surviving skin homografts interchanged between one day old chickens. Repeat experiments have confirmed the observation that the percentage of surviving grafts can be doubled by injecting the recipient daily with cortisone (Cannon and Longmire, 1952)

Green (1952) has emphasized the importance of "stromatization" of homografts following transplantation. To encourage stroma formation in skin homografts, he has filled the local recipient area with a thick coarse emulsion of homologous embryonic lung tissue. He states, "sufficient time has not elapsed to allow a final assessment of the value of the method, but some encouragement is offered by the fact that many 'takes' have occurred and the transplants have remained intact and healthy for a longer period than has characterized the majority of control grafts "

Dukes and Blocker (1952) found that grafts of homologous skin treated with streptodornase-streptokinase persisted 62 per cent longer than did similar transplants untreated with these enzymes on the same animal They suggest that these enzymes destroy the nuclear proteins, released by traumatized cells of the graft, as they are absorbed by the host

The only clinical uses for skin homografts at the present time are. (1) In the rare case of an extensive burn where skin homograft "dressings" may be used during an interim period to build up the patient's general condition or (2) as

a replacement tissue in small vital areas such as the eyelids when autografting is contra-indicated.

When speaking of a skin graft, the clinician not only implies an epithelial covering but also a sufficient accompanying layer of dermal connective tissue to provide a stable foundation for the epithelium. Third degree burns of small or moderate size will usually heal spontaneously by slow marginal epithelial ingrowth. Healed wounds of this type with a thin layer of epithelium growing directly on the underlying scar tissue are unsightly, often interfere with function, and are unstable. For clinical purposes, the dermal tissue is an indispensable part of a skin graft.

Gaillard (Kooreman and Gaillard, 1950) has reported encouraging results from the homografting of fœtal endocrine tissues in man. The tissues are grown before grafting in tissue cultures, by special methods of cultivation. We have confirmed the unsuccessful efforts of others to utilize the skin of the new-born baby as a homograft.

In recent unpublished investigations, we have studied the influence of the age of the donor and the age of the recipient on the survival of skin homografts in chickens (Cannon, Weber and Longmire, 1953).

Previous results from our laboratory and the investigations of others have demonstrated that homografted tissues will survive permanently in a limited percentage of cases when the tissues are interchanged between chick embryos or newly hatched chicks. Homografts between adult chickens, on the other hand, are uniformly unsuccessful.

The "take" of homografts during the embryonic or early post-embryonic period might be explained by (1) The tissues, i.e. skin of the body, have not developed or accumulated sufficient individuality or antigenicity at this age to provoke the destructive reaction in the recipient when transplanted as homografts. (2) The recipient organism at this age has an inadequately developed mechanism to produce and maintain the destructive reaction even when an adequate stimulus is present. (3) Survival of a small percentage of homografts

may result from a combination of (a) a weakened stimulus (from the graft), together with (b) a poorly developed destructive mechanism in the body of the host.

The present series of experiments was designed in an attempt to elucidate the significance of these various factors.

In series A, the age of the skin donor remained constant at one day while the age of the recipients varied (one day to fourteen days). It might be considered that the graft antigen qualitatively remained constant (Although the grafts were grossly similar in size, the quantitative dosage factor of Medawar was disregarded.) Any difference in the survival rate of the grafts in these groups, therefore, should be a result of a variation in the effectiveness of the destructive reaction. The results obtained indicate that this destructive mechanism is not complete until the recipient chick is seven days of age; but at that time it is totally developed and uniformly destructive, even to one day old chick skin.

In series B, the age of the recipient remained constant and the age of the donor varied. We may consider in this series that the potential or capacity of the destructive reaction is constant while the antigenic stimulus from the graft may vary. The results here indicate that skin up to seven days of age has the reduced antigenicity of day old chick skin After seven days the antigenicity rapidly increases until the destructive reaction is 100 per cent by the fourteenth day.

In series C, the age of both the donor and the recipient were varied. Although both the age of the donor and the age of the recipient were within the limits which had produced successful grafts when only one factor was varied, slight simultaneous increases in both donor and recipient ages gave uniformly unsuccessful results. This would again support the theory that there are factors in both the donor skin and the body of the recipient which govern the completeness of the destructive reaction.

The combined results from these three series of experiments would indicate that after hatching certain changes occur in both the recipient and the donor which results in a uniform

tissue incompatibility by the end of the second post-embryonic week

No information has been obtained from these experiments regarding the site of such a "change" in the body of the recipient or of its general nature The "change" may be dependent upon the development of a certain organ or system, or of certain cells, or it may be a total systemic "change" affecting all the cells of the body On the other hand, the "change" that takes place in the donor tissue or graft obviously must be a systemic alteration which can be detected in tissue from many parts of the individual's body and probably represents an individual systemic cellular alteration. This would indicate a period of at least partial individual neutrality (up to about seven days post-hatching) before gene-determined individual specificity becomes absolute

It would seem quite possible that a similar period of individual neutrality may occur in the early life of the human fœtus

As further evidence of the adaptability of "individual specificity" ten healthy adult skin homografts, which had been grafted when donor and recipient chickens were one day old, were removed from the adult host and transferred back to the original donor also grown to adulthood Nine of the grafts survived transfer back to the original donor A limited number of transfers of these healthy adult homografts to a third bird, not related to either the original donor or original recipient have been totally unsuccessful These findings suggest that in the majority of the healthy adult homografts, compatibility with the original donor is maintained even after the graft is adapted to the environment of the new host. Inability to transfer these grafts to a second recipient indicates that the grafted tissue has not become entirely neutral in regard to its tissue specificity

In another group of five adult chickens bearing healthy homografts transplanted when donor and recipient were one day old, repeat homografts were made between the original

donors and recipients. Of these grafts, only one survived. The other four grafts underwent a reaction and slough, usually after a period of healthy "take". There was no effect noticed on the original homograft during the reaction and slough of the second transplant. These findings would indicate that the survival of the original homografts was not a result of the transfer of tissue between two birds with a permanent general tissue compatibility (a "compatible pair"). It would appear that the tissue of the original graft had been accepted by the host during a temporary phase of compatibility and had subsequently become adapted to the new environment Such experimental results lend no immediate assistance to solving the problem of successful homografts in patients but they do give some encouragement to the belief that the successful transfer and survival of living tissue from one individual to another is not an insurmountable problem.

Summary. The use of homostatic grafts in clinical medicine continues to expand in various fields. The improvement of clinical reliability remains the foremost problem. Refinements of methods of preservation and storage that will make these tissues readily available to the general medical profession are also urgently needed.

The homovital graft has little clinical usefulness at present. Several lines of investigation have been mentioned which encourage the view that individual specificity can be modified and that permanent homovital grafting in human beings is not an insoluble problem

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DISCUSSION

ROGERS I would like to add only a few general remarks to Longmire's excellent clinical review For several years I was not c vinced that the so-called acquired immunity reaction applied to human, particularly because we had little or no confirmation the second-set phenomenon in clinical papers Realizing this l of confirmatory evidence, at Bellevue Hospital in New Y City we performed skin homograft transplantation experiments human volunteers As a result of these experiments, we now beli that we have conclusively demonstrated the second-set phenomenon the human In order to determine whether this phenomenon wa systemic rather than a local reaction, the first-set homograft was tra planted to the right thigh of each patient, and after it had been rejec and sloughed completely, the second-set homograft was transplan to the opposite thigh A much more rapid rejection and destruction the second-set graft took place, thereby indicating that the mechan

of the second-set phenomenon and the rejection reaction itself were mediated systemically, in the circulatory bloodstream

At the same time we were making these observations, we also discovered a consistent circulatory and tissue eosinophilia in response to the presence of the *full-thickness* skin homografts This eosinophilia subsides when the very last vestiges of the skin homograft are sloughed It is interesting that a preliminary study of biopsies of the graft and the surrounding host tissue demonstrates that the tissue eosinophilia does not seem to invade the graft proper, but is chiefly confined to the marginal host tissue Within this marginal host tissue, eosinophils can be seen in markedly profuse numbers clogging small host blood vessels and streaming through the host's dermal and epithelial layers We have not conjectured as yet the rôle which the eosinophil plays here Despite the fact that the second-set homograft is rejected more rapidly, the curve of eosinophilia charted for the second-set homograft's behaviour almost mimicked identically in its intensity the curve of eosinophilia charted for the first-set homograft's behaviour Though we have no explanation for this, the close similarity or parallelism of eosinophilia curves for the two sets of homografts seems to indicate that the eosinophil response is a specific response to the presence of the homograft. Dr Hans Roth in Switzerland has reported a similar eosinophilia appearing between the tenth and twentieth post-operative days in patients who received homologous bone grafts

We cannot say at this time whether the response is directed against living or dead tissue, nor is it possible to say whether it has any relation to the eosinophilia seen in response to parasites, allergens, or foreign proteins I cannot help but be impressed, however, by the histological similarity of the breakdown of a full-thickness skin homograft and its concurrent eosinophilia, with that of the ulceration and breakdown of a severe allergic skin test reaction in which numbers of eosinophils are present in marked profusion Because the initial and earliest detectable damage to the tissues in both the rejected homograft and the severe allergic reaction with ulceration is a terminal congestion, stagnation, and gangrene of the end capillaries, the eosinophil may very well initiate these vascular changes Perhaps the chief site at which homograft reactions *per se* take place is in the vascular components of the rejected tissue, or the endothelial living cells themselves The eosinophil or the lymphocyte may liberate the agent which initiates the host's defence reaction against the foreign protein of the graft It is thought that the eosinophil releases histamine at the site of an allergic wheal or severe allergic reaction Whether this is also its rôle at the site of a rejected skin homograft, has not yet been demonstrated Experiments in which antihistamines have been used in an attempt to prolong the life of a homograft have to date been unsuccessful

There is one fact I would like to add to Dr Longmire's report about the homografting of embryonic and fœtal tissues Dr Beverly Douglas of Nashville, Tennessee, has been conducting recent experiments in the grafting of human amnion and chorion to defects in the human In his preliminary report, although he does not claim survival of these grafted

fœtal membranes, he does state that the grafts survive for a period of at least seventeen days

ROB In Dr. Longmire's remarks on blood vessel grafting he said that he thought the new endothelium in the graft grew out from the ends of the host artery I must say I doubt if that is the whole answer I think it is a characteristic of vascular endothelium not to grow out far, in the way skin does, from the edge I think that probably cells deposited from the blood stream on to the surface of the graft form most of the new endothelium—such cells as histiocytes The length of the graft is immaterial to its success, and if there was going to be growth from the end you would expect the length to have considerable importance in the success rate

ROGERS In a recent discussion on arterial grafting, which I attended in New York City, one of the research workers whose name escapes me mentioned that he didn't believe that the length of a vessel was of any importance in its resurfacing by endothelium He believed that a thin layer of endothelial cells quickly resurfaces the lumen of the graft in a manner similar to the extremely rapid rate with which mesothelial cells resurface a large area of denuded peritoneum within a matter of days He felt that this resurfacing took place from the adjoining vessels

ROB Entirely from growth from the ends?

ROGERS Yes

DEMPSTER In Europe things seem to grow more slowly! I have asked various people to show me histological proof of this vascular endothelium growing mysteriously along the graft. I've had negative replies from everyone I have never seen a photograph of endothelium growing out along a graft. I have studied this problem in kidney transplants, the renal artery is anastomosed to the carotid artery of the host. After eighteen days, the kidney and the arterial anastomosis are removed There is no sign of host endothelium growing along the renal artery And if it did, where is it going to stop? I think the supposed endothelial growth may perhaps be an artefact

ROB Figure 1 illustrates this point It shows a human artery graft, a frozen homograft, nine months afterwards, when the patient thrombosed the host vessel, and the graft was patent It shows the centre of this 20 cm long human artery graft, stained for elastic tissue I think the intimal layer particularly interesting because it is so very thick It doesn't look there as if it has grown out from the ends, I think it is probably a deposit from the blood stream

PATE The growth rate probably differs even between New York and Washington! We have now investigated the problem in about 150 artery grafts. Multiple sections taken longitudinally through the grafts demonstrate that endothelium definitely does grow in, but at a very slow rate, in three months, for example, there may not be more than 4 or 5 mm I shall show some pictures of that later

POMERAT In tissue culture of human rib marrow, the rate of growth of endothelial tubes with a basal diameter of about 50 μ is about 2 mm in six days I've made a series of measurements of that

MEDAWAR: Isn't that also the case in the ordinary formation of

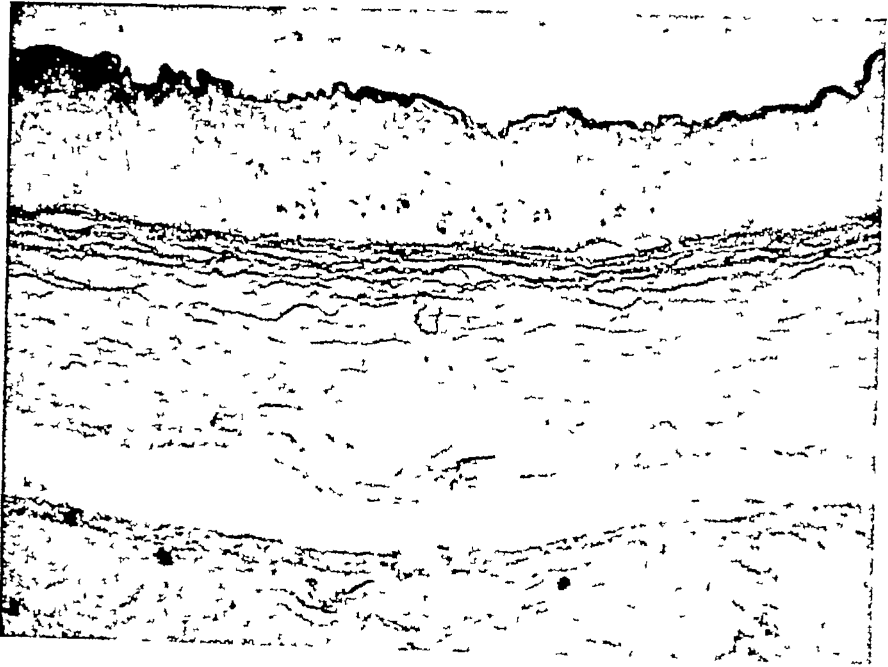


Fig 1 (Rob)

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granulation tissue *in vivo*? Doesn't endothelial tissue grow at just about the rate Prof Pomerat mentioned—so as to form about 2 mm. of granulation tissue in about six days?

ZUCKERMAN What about the peritoneal cells? I was most interested in the observation that they can re-surface vast areas so quickly

ROGERS Dr John H Mulholland in New York City, who is a gastro-intestinal surgeon, has expressed again and again his utter surprise when he has had to re-explore a patient's abdomen and has found that within a matter of only a few days a tremendously large area of denuded peritoneum has been re-surfaced completely with peritoneal mesothelial cells Apparently the proliferative capacity of these cells is highly developed

HUFNAGEL I think that it's most important to realize that it is possible to put in long grafts with successful results It is difficult to get very long segments in animals and most of the patients with grafts of 40-50 cm have (fortunately) not had any complications which would allow one to determine the rate of endothelial growth In animals with grafts of 10-15 cm, which are about the maximal feasible grafts, one will find at the end of sixteen to eighteen months that in the centre there may very well be an area in which, by none of the standard methods, can any endothelium be demonstrated However, near the suture lines endothelium can be readily demonstrated on the graft side The methods for demonstrating endothelium are such that in general the evidence is certainly not conclusive The best way that this can be demonstrated is by silver staining of the interstitial substance, and this, as far as I know, has not been carried out on freeze-dried material

LONG-TERM, LARGE-SCALE, TISSUE CULTURE

WILTON R. EARLE

DURING the last few years much of the work of our laboratory has been concentrated on trying to increase the facility and accuracy with which long-term tissue culture could be used as an experimental instrument In particular, we have been concerned with the practicability of growing, and of using in experimental studies, large-scale cultures of pure strains of both normal cells and of the corresponding cancer cells which might originate from them Today I want briefly to summarize for you certain of the problems encountered and certain phases of the progress made in this study.

The type of tissue culture which was routinely used in our laboratory at an early stage of this study is shown in Fig 1 (Earle, 1943) A strip-shaped explant of tissue about $\frac{1}{2} \times 3 \times 15$ mm in size was planted in a modified Carrel D-3 5 type flask This explant was embedded in a thin layer of solid clot made up of plasma diluted with a mixture of horse serum, chick embryo extract, and balanced saline After clotting of this plasma matrix, 1 0 ml of the same dilute horse serum-chick embryo extract was added as a supernatant fluid layer This supernatant was changed three times weekly At twenty-eight days the cell sheet had usually covered the floor of the flask and was transplanted by removing the sheet from the flask, cutting it into strips, and implanting each strip in a new flask. By this method of culture it has been possible to maintain cell strains in culture over a period of years

At the time this type of culture was in routine use, it had never been possible to grow a culture from a single isolated cell All cultures had to be started originally from tissue fragments or cultures which contained many cells, often

many thousands of cells. Consequently, even when the greatest care was taken in preparing cultures and even when the cell strain was maintained *in vitro* over an interval of years until its morphology became relatively stable and uniform, there could be no assurance that all cells of any culture were comparable in origin and in type (Earle, 1943)

Working on the concept that even the best of our culture media was actually very poorly suited for the growth of

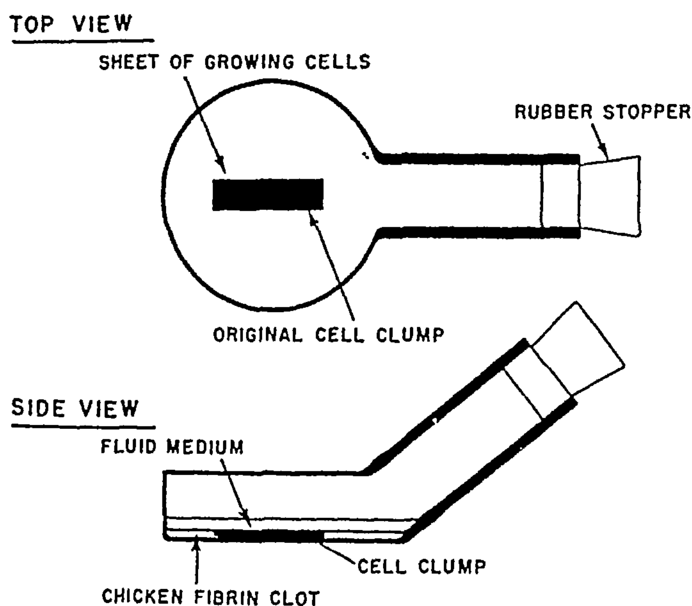


FIG 1 Diagrammatic top and side view of a culture growing in a plasma or fibrin clot substrate in a Carrel D-3 5 type flask.

tissue cells *in vitro* until the medium had been altered or "adapted" by the metabolic activity of living cells, Sanford, Earle and Likely (1948) carried out experiments in which single cells isolated from a strain of mouse sarcomatous fibroblasts, designated as strain L, were planted in capillary glass tubes The capillary tubes served to restrict diffusion of the medium around the cell, and gave the isolated cell a better chance to alter the culture medium directly adjacent, thus allowing it to build up a more optimal zone of altered

or conditioned medium around itself. To give even better adaptation of the medium to the needs of the cells, in some instances the culture medium used was freshly taken from a rapidly growing large culture. In a number of instances experimental conditions were controlled with such accuracy that the isolated cells proliferated, migrated out of the open end of the capillary into the culture medium in the surrounding culture flask, and grew to a large culture of cells.

To date the strain of cells from which the single cell of strain L was isolated has been maintained in culture in an entirely heterologous medium for more than eleven years (Earle, Schilling and Shelton, 1950) The pure strain of cells originating from a single cell has continued to proliferate in culture for more than four years now It is at present used as a standard strain in much of our work

In studies on numerous other types of cells isolated by the method described, many of these isolated cells, both normal and malignant, have been observed to undergo mitosis To date four pure strains of cells have been established and are being maintained in our laboratory (Likely, Sanford and Earle, 1952). These are the strain L sarcomatous fibroblasts, a strain of mouse liver epithelium, a strain of mouse subcutaneous fibroblasts, and a strain of mouse sarcoma cells.* It appears probable that the general method used in the isolation of these four strains of cells can be used with any cell types which can be maintained in rapid proliferation in large cultures

The establishment of pure cell strains raised the question of how to insure that such strains would not be contaminated by extraneous cells accidentally introduced with the culture medium

A most satisfactory nutrient solution for cells of these strains, as well as many others, is a dilute mixture of horse or other serum and an extract made from embryonic tissue. In

*As rapidly as methods for handling these various pure cell strains can be standardized, the strains are being released to other laboratories capable of maintaining and using them The first and second strains listed are already being released

order to eliminate the hazard of contamination of the culture by cells from the serum, in our laboratory the horse or other serum used is routinely filtered by pressure through a Selas ceramic filter* which has a bubbling pressure in excess of 25 lb. The embryo extract, however, could not be filtered without rapid clogging of the filter and great loss of activity. A study of the filtrability of this extract by Bryant and Peppers (Bryant, Earle, and Peppers, 1953) showed that two factors were responsible for the clogging of the filters The first was entirely removed by ultracentrifugation at 45,000 *g;* the second was removed by treatment with a low concentration of hyaluronidase before filtration. Embryo extract may now be prepared in lots of over 1 litre in this way, by the use of such filtered solutions the maintenance of purity of cell strains originated from single cells is insured

In the earlier plasma cultures (Earle, 1943), it had been necessary to provide the cells with a solid surface or a solid matrix on or through which the cells could migrate. While numerous other substrates had been tried, the plasma or fibrin clot had been by far the best, and was generally used (Earle, 1953) This necessity for use of a plasma clot in order to obtain luxuriant cell growth has greatly handicapped tissue culture work The clot frequently clouds up or liquefies, the cultures embedded in the clot cannot be quantitatively separated from it for chemical analysis or for weighing, the chemically undefined nature of the clot has limited exact work in cell chemistry and in cell nutrition, the cell clumps are limited to small size.

As the culmination of a long series of experiments to find a substitute for this plasma clot, Evans has shown that excellent cell proliferation of many cell types may be obtained by growing the cells in the usual horse serum-chick embryo extract fluid medium under a sheet of finely perforated cellophane (Evans and Earle, 1941, Earle, Evans, Edward and Duchesne, 1949) This cellophane sheet helps to hold the

*Selas Filter Company, Erie and D Streets, Philadelphia, Pennsylvania, U S A

explant and the cells of the young culture in position so that agitation of the culture fluid does not easily wash them away. The roughness of the cellophane sheet around the edge of the perforations, and the extension of cell strands through its perforations on to the opposite side of the sheet, causes the cellophane to offer a far better surface of mechanical attachment for the cells than does the glass of the culture flask. It is probable that the perforated cellophane sheet also acts in the same way as the capillary tube in the single cell culture and by somewhat restricting diffusion allows the cells of the young culture to build up a zone of modified or "conditioned" culture medium around themselves

With the plasma clot type of cultures, even from established cell strains, cell sheets which covered the floor of a flask 33 mm in diameter were considered excellent, and would possibly contain 10 mg wet weight of cells, often the cell sheets were much smaller With many types of cells planted as single large explants under perforated cellophane, cultures of far larger size can be grown For instance with the pure strain L cells, cultures which covered the floor of an 80 mm. diameter flask were rapidly obtained (Fig 2). At the present time, cultures of all rapidly growing cell strains maintained under cellophane are grown in culture flasks having a floor area of 60 cm ^{2} (Earle and Highhouse, 1953)

One study now in progress which involves the use of these large cellophane substrate cultures may be of particular interest. In collaboration with the personnel of the Tissue Bank of the U.S Navy Medical Center, Evans and Waltz, in our laboratory, have been carrying on a study of the growth of human skin cells in culture. Already one strain of mixed cells, including both skin epithelium and fibroblast-like cells, has been maintained for more than one and one-half years This strain was originally started from a fragment of skin about 3×15 mm in size At one time we were able to use in one experiment cultures which had an area of over 1600 cm.^{2} of these skin cells

With this mixed strain of cells, as with other rapidly

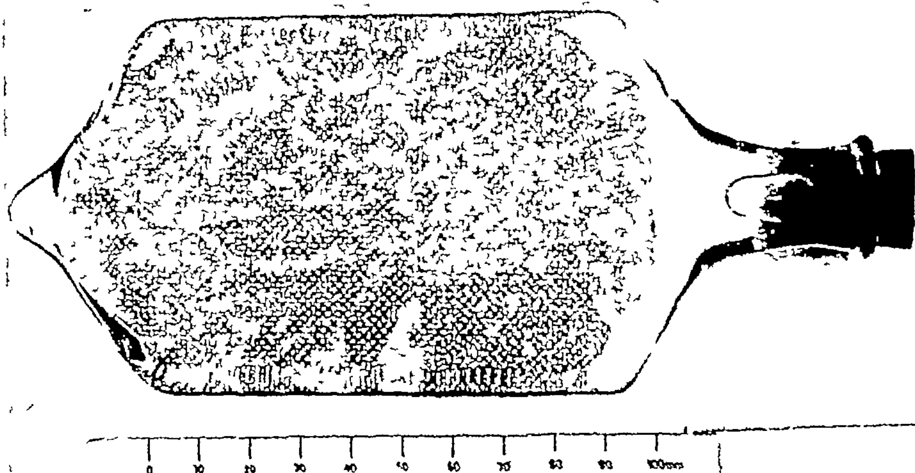


Fig 2 Twenty-eight day old culture from an established strain of mouse liver cells growing under a sheet of perforated cellophane The preparation has been fixed and stained in iron hæmatoxylin The stained area is the cell sheet and the small clear circles through it are the cell free areas of the cellophane perforations Cell density over the sheet is irregular due to wrinkling of the cellophane This culture flask has a floor area of approximately 60 square centimeters

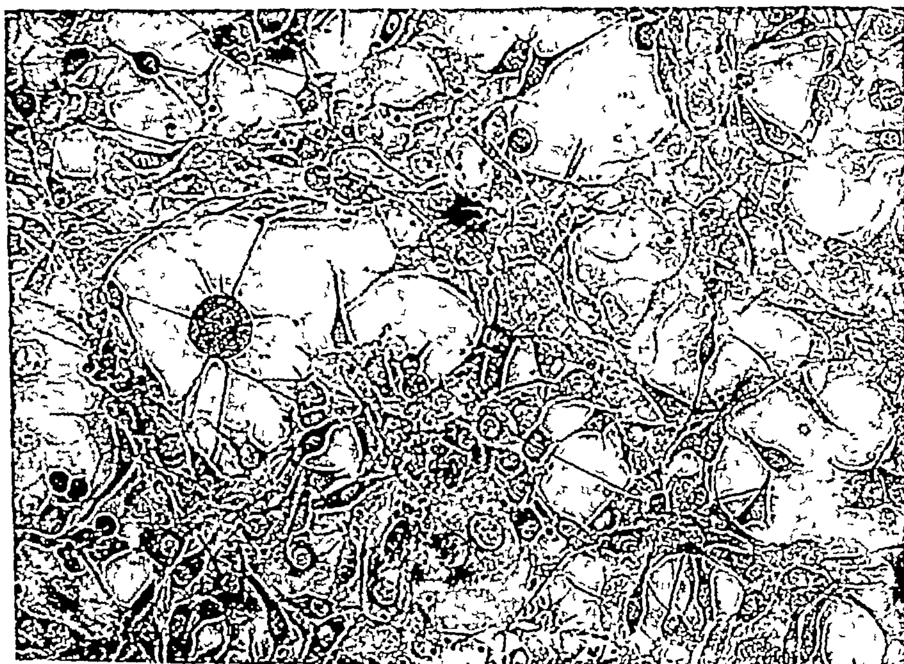


Fig 3 Area of a living culture of pure strain L mouse subcutaneous fibroblasts, to show the morphological variation which may occur in cells of such a pure strain grown from a single cell Note in particular the very large rounded up cell and the extremely thin sheetlike binucleate cell $\times 133$

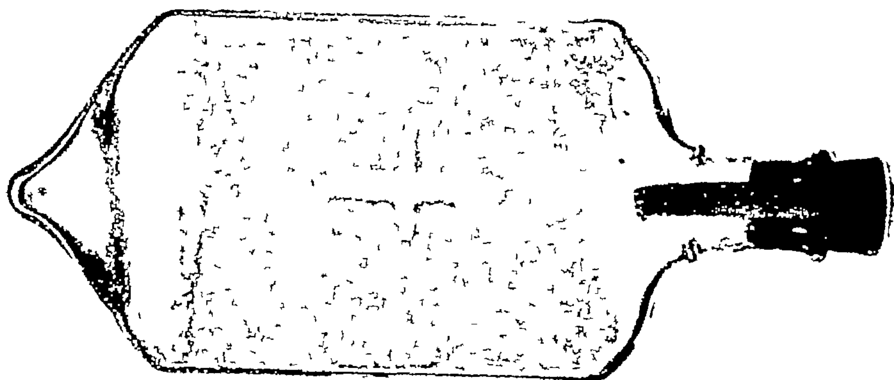


Fig 4 Living unstained sheet of strain L fibroblasts growing on the glass surface of the culture flask The clear area near the centre was scratched through the sheet by means of a spatula Magnification × approx 100

Fig 5 Photomicrograph showing the appearance of a matrix made up from pyrex glass helices

proliferating long-term strains, the difficulties of cell identification and classification must be emphasized. The characteristic architecture of the tissue is lost, the epithelium of the skin migrates out first as epithelial sheets, the cells appear to lose their intercellular bridges; the sheets break up, the cells become more migratory, more elongate, far more fibroblast-like

Similar and no less radical morphological changes occur with long-term cultures of other highly specialized cells such as liver epithelium (Evans, Earle, Wilson, Waltz and Mackey, 1952) The architecture of the lobule is lost, the cells migrate out from the explant as an epithelial sheet; this ultimately breaks up into individual more migratory cells, rounded or amœboid in contour. Even with the cells of pure strains which have originated from a single cell, substantial variation in morphology occurs (Fig. 3).

Without doubt more critical criteria are needed for the identification and ultimate characterization of many cell types under the wider range of experimental conditions which may be brought about in long-term tissue cultures. At present it appears that such ultimate identification and characterization of a cell type must rest on morphological and physiological criteria established from pure cell strains under carefully established and controlled experimental conditions

By shaking and scraping an established and rapidly proliferating cellophane substrate culture, many cells come loose from the substrate and form a suspension in the supernatant fluid (Earle, Evans and Schilling, 1950). This suspension of isolated cells and small cell clumps may be used to plant new cultures of even larger size From such suspensions, cultures of 60 cm ^{2} area and even larger may be routinely obtained, and from each such culture relatively large volumes of extremely dense cell suspension may be prepared By using such a dense cell suspension as an inoculum, it has been possible to eliminate the cellophane substrate (Shannon and Earle, 1951) and to obtain luxuriant cell proliferation directly on the floor of the pyrex glass culture flask Thus with pure

strain L, and other rapidly proliferating cell strains, approximately 100 mg wet weight of cells can be obtained from the growth on the glass floor of a flask of 60 cm.[2] floor area (Fig. 4). This requires from four to sixteen days depending on the proliferation rate of the cells and the density of the inoculum

At the present time, our stock cultures of rapidly growing cell strains are all planted by this cell suspension procedure and are carried under cellophane or on glass substrates in 60 square centimetre flasks From such cultures, in a number of instances we have been able to obtain lots of from 1–10 g. wet weight of cells for chemical study. Even with present facilities, by discontinuing other work, and by using a rapidly proliferative cell strain such as pure strain L or the pure liver epithelium, probably 100 g wet weight of cells could be grown in such flasks within a period of thirty to forty days.

This method of planting large cultures from cell suspensions is not limited to established cell strains By passing tissues of nine day chick embryos through progressively finer sieves down to 150 mesh, Shannon (Shannon, Earle and Waltz, 1952) has been able to obtain similar large sheets of cells from cell suspension inocula of even these fresh tissues These cultures showed many varieties of cells, including muscle fibres, epithelium, pigmented cells, fibroblasts, etc. Such cultures have been maintained without transfer for more than six months

At the time we were routinely using plasma clot cultures (Earle, 1943), attempts had been made to use change in diameter or change in area of the culture as an index of rate of cell proliferation. The attempts were ultimately abandoned since it was impossible to obtain substantial numbers of accurate replicate cultures and since changes in the architecture of the cultures under the action of the experimental conditions used in some instances caused demonstrable errors far in excess of 1000 per cent With the large glass and cellophane substrate cultures however, coarse suspensions of cells shaken or scraped from the substrate can be passed

through fine platinum alloy sieves, down to 150 mesh (Evans, Earle, Sanford, Shannon and Waltz, 1951). The extremely uniform suspension so obtained consists of isolated cells and small cell clumps These can be separated by gentle centrifugation, washed, and resuspended in a balanced physiological saline solution. Then, by means of a specially designed burette, aliquots of the suspension can be buretted into each of a large number of culture flasks to start new cultures. For instance, from 3 60-cm.^{2} area stock cultures of the pure strain L cells, 100 ml of suspension may be routinely prepared, and from this, 100 15-cm ^{2} area experimental cultures may be planted within three hours. By this method of planting it is now practicable to obtain from rapidly proliferating cell strains large numbers of accurately replicate cultures.

Concurrently with the development of methods for preparation of large numbers of replicate cultures, methods were worked out by which the numbers of cell nuclei in such cultures, or in aliquots of the cell suspension used to plant them, can be determined The cell nuclei can be quantitatively removed from the glass substrate, and the nuclei in aliquot volumes of the suspension enumerated in a standard hæmocytometer (Sanford, Earle, Evans, Waltz and Shannon, 1951) With satisfactory cell types replicate cultures can be planted, subjected to various experimental conditions for from one to three weeks, and their nuclei enumerated; from the data so obtained, curves showing changes in the nuclear population levels of the cultures under experimental study can be plotted. At the present time with the pure strain of L cells, for instance, statistical examination of recent experiments shows that in the preparation and planting of replicate cultures from such cell suspensions and in the enumeration of their nuclei, the overall error is less than 10 per cent with 95 per cent probability of accuracy. Furthermore, with this method of preparation only nuclei normal in morphology and staining reaction are enumerated; necrotic or disintegrating nuclei do not stain. To date, however, we have not been able to apply this method to cells which lay

down a fibrous intercellular matrix, or to cells of one strain of chick fibroblasts which had extremely small and achromatic nuclei

As far as I know, at the present time, every mammalian tissue cell strain grown for an extended period of time such as a year, has been grown in a nutrient medium which contained a serum, or a tissue extract (usually from embryonic tissue), or a mixture of the two. In our own experience, a mixture of serum, such as horse serum, and embryo extract, such as is obtained from nine-day chick embryos, has given a most favourable medium for the rapid, long-term proliferation of many types of cells. As more accurate control of experimental culture conditions becomes necessary and as need for larger cultures develops, there has been an ever increasing need for chemically defined culture media Studies are now underway in many laboratories in attempts to obtain such experimentally controlled media.

Time does not allow a detailed consideration of this field, but rapid progress toward such media is being made in many laboratories In our own laboratory for instance, Westfall, Sanford, and Evans, and their associates, have been attempting to fractionate embryo extract and horse serum nutrient mixtures, and to substitute these fractions by chemically defined materials (Sanford, Waltz, Shannon, Earle and Evans, 1952; Evans, Shannon, Bryant, Waltz, Earle and Sanford, 1953, Westfall, Peppers, Sanford and Earle, 1953; Westfall, Peppers and Earle, 1953) These mixtures have been studied on pure strain L fibroblast cultures planted from washed cell suspension inocula From these as yet incomplete studies it already appears possible to eliminate entirely the non-dialysable fraction of the embryo extract from the media, and to eliminate also the ultrafiltrate fraction of the serum. In the mixture currently under study, cultures show a rate of increase of nuclear proliferation which equals that of cultures in the standard horse serum-chick embryo extract mixture routinely used

The development of the nuclear enumeration procedure

for estimating proliferation also allowed us for the first time to study quantitatively the relationships between the volume of culture fluid and the number of cells necessary in the inoculum (Earle, Sanford, Evans, Waltz, and Shannon, 1951). For example, with 2 ml. of highly favourable standard culture fluid such as dilute horse serum-chick embryo extract, an initial inoculum of strain L cells equivalent to 100,000–500,000 nuclei resulted in proliferation of all cultures planted; an inoculum of 10,000–50,000 gave only 64 per cent of the cultures proliferating, while an inoculum of cells equivalent to 600 nuclei gave no proliferation. At between 6,000,000 and 10,000,000 nuclei, further increase in the population of the culture ceased; at this level population often actually lessened due to necrosis and cell disintegration. It appears that accurate quantitative experimental studies can be carried out only when this critical relationship of cell number to the nutrient fluid volume is carefully controlled.

While the use of plane glass substrates, such as the glass surface of the flask, had allowed a great increase in the amount of cells which could be grown in culture, the plane substrate placed a practical limitation on the amount of cells which could be grown in the individual culture. An attempt was therefore made to build up a three-dimensional matrix, on the surfaces of which the cells could attach themselves and through the interstices of which nutrient fluid and carbon dioxide-air mixtures could be periodically recirculated (Earle, Schilling and Shannon). Both folded cellophane matrices and matrices which consisted of masses of 1/8 inch diameter pyrex glass helices were tried (Fig. 5) The usual dilute horse serum-chick embryo extract nutrient fluid was used and the cultures were inoculated with suspensions of pure strain L cells, and of strain 721 pure mouse liver epithelium. In all the cultures tried with these types of three-dimensional substrates, Schilling and Shannon could demonstrate cells adherent to the matrix surfaces, and the cell population, as determined by nuclear enumeration, rose far above the original inoculum level Thus in one culture on

glass helices the final number of nuclei enumerated rea
174,282,200, estimated as in excess of 700 mg wet weigh
cells, in another culture, on cellophane, the number of nu
enumerated was 286,310,000, estimated as in excess
1145 mg. wet weight of cells.

The question arose as to the influence of the rate of circ
tion of nutrient medium over the cells. Since the variable
the three-dimensional cultures were too complex for sin
experimental analysis, the study of the influence of velo
of flow was undertaken on pure strain L cell suspen
inoculum cultures planted in culture tubes of 22 mm. inte
diameter and specially designed for nuclear enumera
studies. After planting, these tubes were kept rotating
incubating them in a multiple drum rotating or roller t
unit (Gey, 1933, Earle, Schilling, and Bryant, 1953). T
unit had eight drums, the rates of rotation of which w
9·37, 18·75, 37·5, 75, 150, 300, 600 and 1200 revolutions
hour respectively. Control cultures were also carried w
cells in stationary tubes and in stationary T-15 flasks. '
results are shown in Fig. 6.

The significant point of this study (Earle, Schilling, a
Bryant, 1953), so far as our present subject is concerned
that the velocity of rotation of the tube, which controls
linear rate of flow of the nutrient over the inner surface
the tube, was a major factor in controlling the number
cell nuclei in the culture By increasing the rate of rotat
of the tube from the 6 to 9 revolutions per hour usually u
with the roller tube drum (Gey, 1933) to 600 revolutions
hour, the level of nuclear population was raised more th
80 per cent. Within the range of velocities studied the optim
rate lay between 150 and 1200 revolutions per hour. Wit
this range, the population levels were so close together th
no one culture rotation-velocity can be considered as definit
superior over the whole culture period.

In spite of these high velocities of flow it appeared that m
of the cells were adhering to the surface of the glass tube

In all the preceding studies a solid substrate to wh

the cells could adhere was used. Earlier unpublished attempts of ours to demonstrate cell proliferation in cultures in which the cells were freely suspended in a fluid culture medium and not adherent to some substrate had all failed. In spite of these failures, the frequent occurrence of dense cell-suspensions in peritoneal ascites associated with abdominal carcinomata

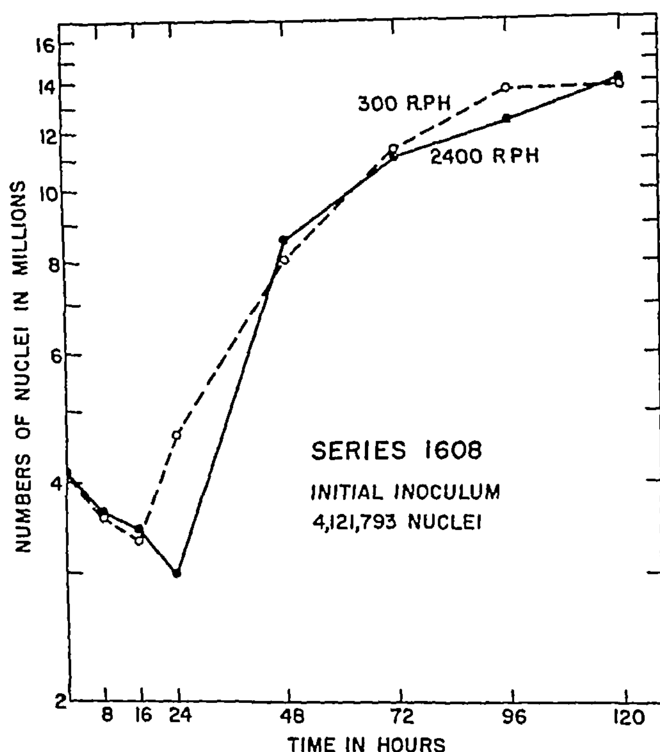


FIG 6 Curves showing increase in population of fluid suspension cultures rotating at 800 and at 2400 revolutions per hour

suggested that cells might be made to live and proliferate in fluid suspension *in vitro*. The growth, by Gey, of cells of a lymphoblastic mouse tumour in "tumbling tube" cultures *in vitro* (Gey, 1953—personal communication) has also suggested this possibility, although there was a definite possibility that cell proliferation in fluid suspension *in vitro* might be a unique characteristic of cells of the lymphoblastic series There

were, therefore no conclusive data to show that the fixed tissue cell, such as the fibroblast, could survive and maintain proliferation in culture *in vitro*, except when attached to a surface substrate

While the solid substrate culture could certainly be developed to an extremely large size, the advantage of being able to eliminate the surface substrate entirely was very great It was essential in the development of the large-scale culture that a better understanding of the rôle of the substrate in the survival and proliferation of the fixed tissue cell be obtained

Since a cell freely suspended in a circulating fluid would be washed on all sides by that fluid, the adjustment or "conditioning" of the fluid to the cell would probably need to be extremely critical in order to allow proliferation To achieve this, a heavy cell suspension inoculum was used in each rotating tube culture. Since our earlier unsuccessful study of fluid suspension cultures had been made with cells of pure strain L fibroblasts, cells of this strain were used. The usual fluid medium of dilute horse serum and chick embryo extract was used, with about 0·1 per cent of methyl cellulose* added to increase very slightly the viscosity of the fluid and so assist in maintaining the cells in suspension The rotating drums were increased in velocity so that the most rapid was rotating at 2400 revolutions per hour.

This attempt to proliferate the strain L cells in fluid suspension has been successful (Fig 6) After an initial period apparently required by the cells to adapt or "condition" the fluid culture medium, the cells established and maintained a state of rapid proliferation during the course of the experiments In cultures of intermediate and higher rotation velocities there was no evidence of cell attachments on the surface of the glass tube with intermediate velocities of rotation the cells aggregated in small granular or spherical masses from 0·1 to 0 5 mm in diameter suspended in fluid; with higher velocities of rotation the cells floated as indepen-

*Methocel, 4000 cps, Dow Chemical Company, Midland, Michigan, U S A

dent single cells and as cell aggregates of up to about ten cells.

The results presented here show how this pure L strain of fixed tissue cells has been carried by a series of steps from culture in a plasma matrix to culture on a glass surface substrate, and has finally been successfully proliferated in fluid suspension culture For this cell strain at least, the results obtained indicate that the solid substrate previously necessary for culture of fixed tissue cells can be entirely eliminated by accurate control of culture conditions Instead the cells can be grown in fluid suspensions under conditions closely comparable to those now used in large-scale growth of bacteria and fungi The nature of the variables involved and the control of these variables already achieved appears to justify the expectation that comparable methods may be used for other types of tissue cells

Already it appears probable that within the very near future, as the need arises, at least some types of normal and malignant animal tissue cells can be grown routinely in very large scale cultures. It is my belief that the time is already at hand when consideration should be given to the implications, and the applications, of such massive growth of animal cells *in vitro*.

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TRANSPLANTATION OF PRESERVED NON-VIABLE TISSUES

JAMES W. PATE

THE use of certain homogenous grafts is now an accepted clinical procedure which is rapidly gaining in popularity Attempts to graft preserved tissues date back centuries, but until the last decade these attempts have been generally unsatisfactory.

There is a definite need for such grafts in modern surgery This is emphasized in the field of vascular surgery, where autogenous grafts of some major blood vessels are impossible to procure. In orthopædic surgery, the use of preserved homogenous grafts shortens operative time, decreases trauma to the patient, and eliminates pain from the post-operative donor site. Homogenous grafts also make possible extensive spinal fusions and other procedures requiring large quantities of bone which are impossible to procure from the patient. Grafting of homogenous skin may be life-saving in a severely burned patient Autogenous cornea are impossible to obtain—as, frequently, are autogenous nerves. Other grafts, such as thyroid, adrenal, cartilage, and tendons are needed The practical aspects of homogenous grafting demand a satisfactory method of preserving or "banking" the grafts from the time of donation to the time of the use

The ideal preserved graft is one which, after storage and implantation, immediately *and* permanently fulfils all the normal physiological functions of the replaced tissue In the light of present knowledge of basic cell function, these requirements cannot be met. Since these standards are not fulfilled today, the most acceptable method of "banking" tissues, in our hands, is based upon satisfactory biomechanical function

The selection of a method of preserving homogenous grafts should depend primarily on the function expected of such a graft after implantation. Homografts can be grouped into two large categories, depending upon these functional requirements As suggested by Longmire, we shall name these the "homostatic" and the "homovital" The homostatic graft's function is mechanical, and includes such tissues as bone, cartilage, blood vessels, tendons, fascia, ligament, and possibly other mesenchymal tissues The function of the homovital graft depends on viability of the cells, with the resultant ability to synthesize, detoxify, secrete, or excrete. This group includes endocrine organs, kidneys, muscle, liver, heart, gastro-intestinal organs and nerve cells.

The fate of homografts after implantation has been intensively studied by many investigators Many lines of attack, such as desensitization, tissue extraction, drug treatment and irradiation, have been followed in an attempt to produce permanent survival of the cells. The one universal result of these studies, however, has been the almost uniform report that adult homografts, placed in their normal location, all die at some time after implantation. There are certain exceptions, such as fœtal tissues in the anterior chamber of the eye, but, in general, all homogenous grafts are dead soon after implantation This dead tissue is removed and replaced by the recipient

The use of homografts in surgery today, however, is not as hopeless as this "homograft reaction" would indicate. All tissues of the homostatic group have a primary mechanical function, and should not demand viability, *per se*, in order to function satisfactorily

To see what may be accomplished by the use of non-viable grafts, we shall discuss a few specific tissues which have been investigated From the standpoint of the surgical use of the graft, certain criteria for the structural function of such homostatic grafts may be established (a) For an artery or vein, the primary requirement is that the graft transmit an adequate amount of blood over a prolonged period without

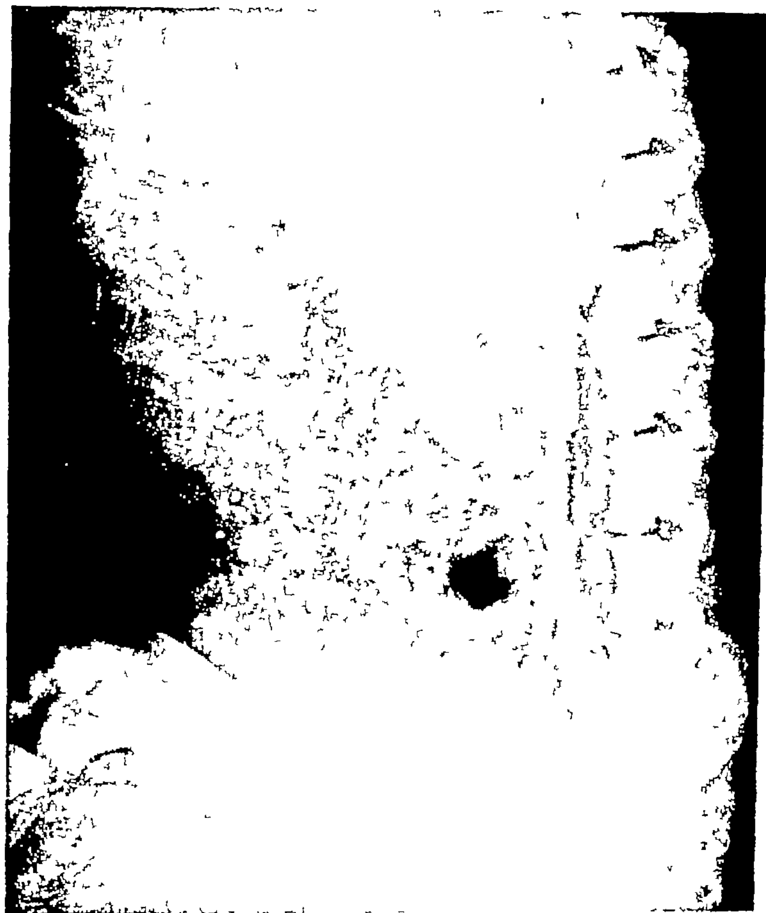


Fig 1 Aortogram of dog, one year after implantation of freeze-dried aortic graft, stored for three months

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and (d) freeze-drying (Kreuz *et al.*, 1951, Marrangoni and Cecchini, 1951; and Pate *et al.*, 1952–53)

These methods may be grouped into (a) Those where viability is maintained These include fresh homografts, nutrient-media grafts, and rare cases of frozen grafts (b) Those where viability is not maintained but the physical and chemical structure is not seriously altered. These include most of the frozen grafts and all of the freeze-dried grafts. (c) Those where viability is lost, but other factors, such as protein structure, are seriously altered This group includes the chemically fixed, merthiolate stored, boiled, and autoclaved tissues and will not be discussed further, because of the unknown variables introduced.

Non-viable arterial grafts were used as early as the first decade of this century. The study of viability in arterial grafts has been vigorously pursued. As a basis for discussion, we shall use the studies from our own laboratories (Pate *et al* , 1952–53; Pate and Sawyer, 1953). We have implanted about 150 aortic grafts in the dog, using fresh homogenous, nutrient media homogenous, frozen homogenous, freeze-dried homogenous, and freeze-dried heterogenous grafts All these grafts have been placed in the abdominal aorta using identical technique Storage time has been controlled so as to be comparable in all groups except in the fresh grafts Evaluation of the grafts has been made primarily on the ability of the graft to carry blood, i e. on the gross neuromuscular function of the dog, the colour, temperature, presence of pulsations, aortograms, and the gross appearance of the graft at autopsy (Table I). In addition, many of the segments have been submitted to extensive microscopic studies, elasticity studies, tensile strength determinations, electric potential difference measurements, tissue culture, histochemical and chemical analysis.

The ability of the graft to carry blood without deleterious effects on the recipient is roughly comparable in all the groups and all are acceptable. However, the freeze-dried homograft (Fig. 1) is notably without rupture, hæmorrhage or thrombosis.

This is apparently confirmed by 14 human grafts followed for only seven months or less Microscopic study has indicated that the freeze-dried homograft is re-endothelialized faster, in general, is more acceptable, with less scarring and fibroplastic proliferation, than any of the other types of grafts

In an attempt to elucidate the mechanism underlying the better results in the freeze-dried graft, several interesting

Table I

STATISTICS OF 185 AORTIC GRAFTS IN DOGS

Time of storage of freeze-dried grafts was from one week to one year, with a mean of six months

	Homografts		Heterografts
	Fresh	Freeze-Dried	Freeze-Dried
1 Number	30	65	40
2 Mortality	4(13%)	1*(2%)	3(8%)
a) Hemorrhage	2(7%)	1*(2%)	3(8%)
b) Thrombosis	2(7%)	0	0
3 Thrombosis, Nonfatal	4(13%)	2 (3%)	3(8%)
4 Gross & Microscope Appearance	-	-	(32)⁺
a) Excellent	17(57%)	60(92%)	15(47%)
b) Good	5(17%)	2(3%)	11(34%)
c) Fair	3(0%)	1(2%)	2(5%)
d) Poor	1(13%)	1(2%)	1(3%)
e) Failure	4(13%)	1*(2%)	3(8%)
5 Acceptable Function	26(87%)	64(98%)	37(92%)

* Same case, no vacuum storage

+ Total autopsies to date Remaining 8 cases are living without evidence of abnormalities

studies have been done Measurements of the electric potential difference (Sawyer and Pate, 1958*a*, *b*) across the walls of vessels have shown that dying, injured or degenerating cells have positive (injury) potentials on their intimal surface in relation to the adventitia These positive intimal potentials attract the negatively charged blood cells and a thrombus is formed on the vessel wall This is in contrast to the normal vessel, where the potential at the intima is negative; and to the freeze-dried graft which, being dead, is incapable of

maintaining any potential The studies have shown that there is a direct correlation between living (injured?) cells in an arterial graft and the predisposition to thrombosis, and conversely, that the absence of living cells in a graft is correlated with an absence of electrical potential and a reduction in the probability of thrombosis. Freeze-dried homografts of the aorta have been completely crushed through their entire length in numerous experiments, without the

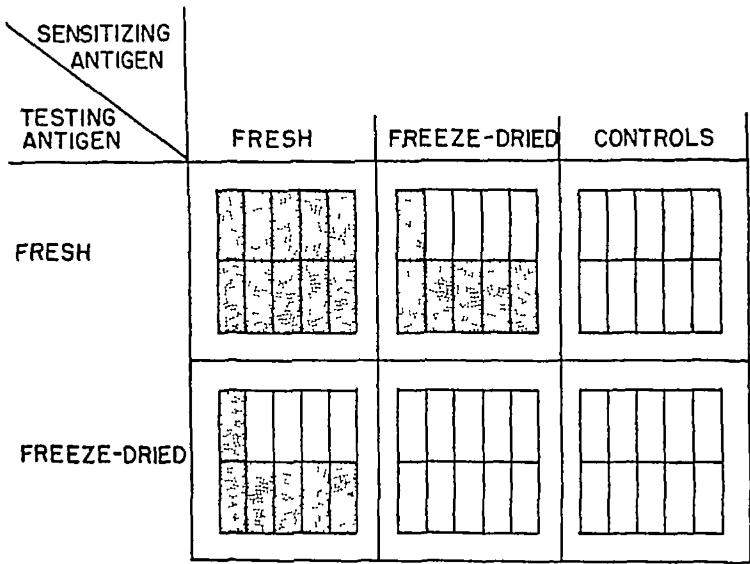


FIG 2 Precipitin tests on rabbits sensitized to crude aortic extract Each horizontal block represents one animal, each vertical block intensity of reaction.

production of thrombi This has been true of no living graft studied, or even of the normal aorta

We have been able to demonstrate that the process of freeze-drying and subsequent reconstitution and extraction in water or physiological saline removes some of the protein from the graft Precipitin tests in the rabbit suggest that some of the antigenic properties are also removed (Fig 2), and it seems quite possible that the two phenomena are related This may be a factor in the decreased scarring and

fibroplastic response observed in the recipient of the freeze-dried grafts. It may also help to explain the fact that by freeze-drying and reconstituting in large volumes of saline, arteries from pigs have given acceptable results when implanted into dogs

These desirable properties (removal of electromotive force and reduction of antigenicity) in the freeze-dried grafts

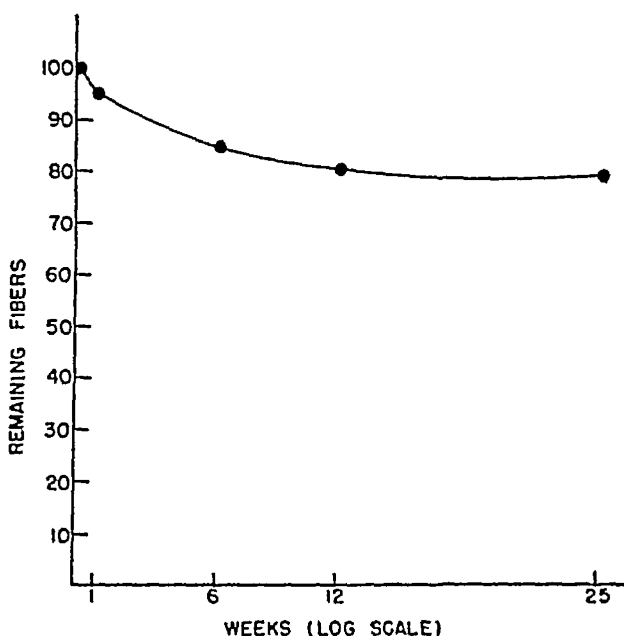


FIG 3 Rate of disappearance of elastic fibres (in percentage) after implantation of fresh and freeze-dried aortic grafts

would both be impossible in the presence of living cells. We have become convinced that viability *per se* in arterial grafts, is not only unnecessary but undesirable

As an example of other comparisons between viable and non-viable aortic grafts, let us consider elasticity. It has been said that, from a clinical point of view, the elastic properties of the aorta are far more important than the histological appearance (Krafka, 1939). It has been shown that rigidity in an artery predisposes to aneurysm formation and rupture,

so that the retention of elasticity is desirable. If one takes multiple random sections through an arterial graft and its donor vessel, and actually counts the number of elastic fibres in the thickness of the vessel wall, the number of fibres remaining after varying periods of implantation is found to decrease. Fig. 3 shows such a curve, based upon approximately 3400 counts from about 1100 sections from 70

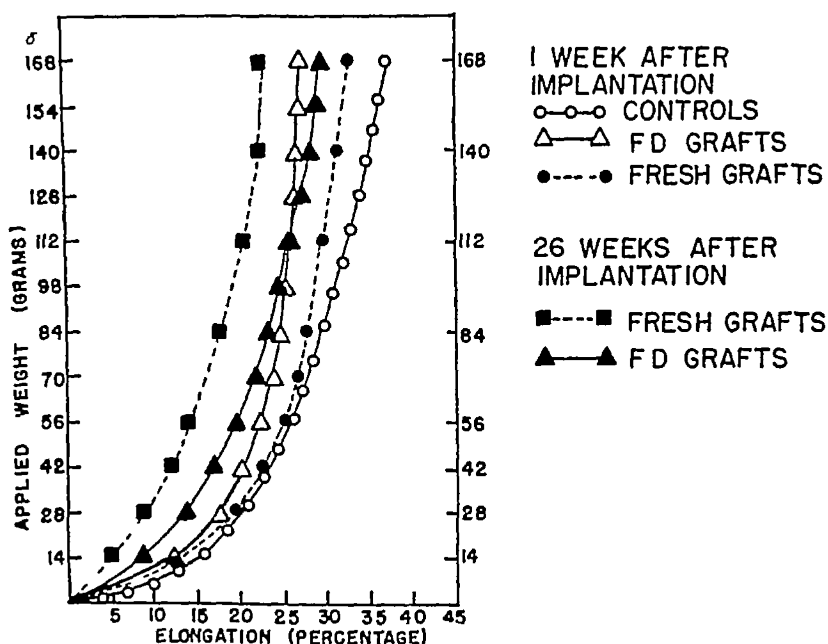


FIG 4 Tension *vs* elongation curves of aortic grafts at various times after implantation

aortic grafts Curves for both freeze-dried and fresh homografts are not significantly different. Microscopic changes in the fibres are comparable in both groups.

Strips of known cross-sectional area may have constantly increasing tension placed on them, so that their elongation gives a measurement of their elasticity. Fig. 4 shows a series of such curves. It is evident that freeze-dried grafts are not only elastic as are fresh homografts, but give curves nearer the normal. Mathematical analysis of such curves

shows that the loss of elasticity after implantation is well correlated with muscle degeneration, fibrous replacement, and peri-adventitial fibrosis Here again we see an area in which the non-viable graft fulfils our practical desires in a more favourable manner than the live fresh homograft

The first record of transplantation of bone grafts which we may be reasonably sure were non-viable was by Ollier in 1867. He reported the use of homogenous bone which had been stored below −16°C Many papers concerning the use of bone grafts have appeared since that time (Inclan, 1942; Bush and Garber, 1948, Herbert, 1951, Wilson, 1951). It is noteworthy that these results are roughly comparable with those obtained from fresh homografts In our laboratories, there has been an extensive comparison of the results in animal experiments of grafting of bone preserved by various techniques and fresh autografts (Kreuz *et al.*, 1951; and Turner unpublished) These studies have failed to demonstrate any superiority of the viable over the non-viable bone grafts. The freeze-dried grafts have been satisfactory and have compared favourably with the other types of grafts. Whether these results will be duplicated in the human patient must be demonstrated by a long-term study. However, short-term observations of over 100 freeze-dried grafts have roughly paralleled the experimental findings in the animal.

These results compare with those of other investigators in that· (a) There is no remarkable difference in the results from viable and non-viable homografts, and (b) non-viable bone serves as an "osteogenic stimulus" in spite of the fact that it is dead.

It is almost universally appreciated that all homogenous skin grafts die and slough from the wound surface at varying times after transplantation Nevertheless, the use of skin homografts has a very real place in modern surgery. In the treatment of extensively burned patients the nearest approximation to the ideal dressing is a skin graft, be it autogenous or homologous (Saunders and Moore, 1950). Homologous skin may be life-saving in such cases (Hyatt, 1953) Well-

preserved non-viable skin may fulfil our basic criteria by decreasing fluid, electrolyte, and protein loss, and by alleviating pain, infections and fibroplasia. Initial clinical results with freeze-dried skin suggest fulfilment of these criteria, and that skin acts as a good physiological dressing

The use of merthiolate-stored, frozen, freeze-dried, boiled and other non-viable cartilage grafts has paralleled the use of bone These non-viable grafts are widely used with acceptable results in spite of the fact that they are dead.

Theoretically, this concept of non-viable homostatic grafting may be extended to other tissues, such as· fascia, tendons, œsophagus, trachea, cardiac valves, nerves, ureters, common bile ducts, dura and possibly cornea. Early experimental results have, in some of these, indicated that the theory may correlate with practical results. It must be determined if these grafts will fulfil their function while being replaced by the recipient.

Conclusions

1. The use of homostatic grafts has a definite place in modern surgery.

2 The necessity of viability in these grafts is open to serious question

3. Experimental studies in animals indicate that at the present stage of development, viability *per se* is actually undesirable in arterial grafts and this seems to be confirmed by short-term human cases.

4. By the utilization of non-viable grafts, various manipulations, such as freeze-drying and extraction, may be carried out. Such lines of attack on homografting may lead to radical improvements in technique of storage and in the results of homostatic grafts, and possibly allow satisfactory heterostatic grafting.

The opinion and statements in this paper are those of the author and should not be construed as official or as necessarily reflecting those of the Department of the Navy or of the Naval Service at large

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DISCUSSION

ROB Did you measure the electrical intimal potentials of frozen grafts as well as the freeze-dried and fresh aortic grafts?

PATE We did not do enough of those to draw any definite conclusions After short periods of storage, they seemed to follow the fresh grafts, but after longer periods, the freeze-dried

LONGMIRE What is the longest period that these freeze-dried aortic grafts have been followed?

PATE In the dog they have been followed for about two years, in the human for periods of about seven months

LONGMIRE Do you ever find any calcium deposits in these grafts?

PATE We have done calcium determinations on a few and found them within normal limits We can see no evidence for it

LONGMIRE H ve you found calcium deposits in other types of grafts?

PATE We haven't, surprisingly enough We have seen a thickening of the intima which resembles calcification grossly, but on chemical examination is not calcium

ALLGOWER Are you sure you kill all the cells by freeze-drying? There have been reports by Webster and other plastic surgeons of the use of skin which was freeze-dried and which persisted afterwards

PATE We have done multiple tissue cultures and have not been able to get any live cells out We feel sure the cells were not alive After as early as forty-eight hours of implantation there are no cellular elements at all present in the graft

PARKES How did you reconstitute the freeze-dried tissue?

PATE We simply put it in water or physiological saline for about thirty minutes

MEDAWAR Are fresh bone autografts "static" grafts, in Longmire's sense? Do you think they are completely replaced by host tissue?

HYATT We think that fresh bone autografts, while replaced in the same static manner as are the homogenous grafts, are somewhat more acceptable to the host from the clinical standpoint In our experimental work* we did not investigate "chip grafts" However, in the patient we make a "boneburger" which is a patty of bone chips It resembles a "hamburger patty" in physical appearance Our initial clinical observations lead us to believe that the rate of revascularization of the "boneburger" is satisfactory

LONGMIRE I would like to ask your opinion of Hatcher's observation that the viable cells in a bone graft may participate in the early phases of healing

HYATT As I understand Hatcher's observation, he bases his interpretation of viability upon the basophilic staining portions of the cell

*Hyatt, G W, Turner, T C, Bassett, C A L, Pate, J W and Sawyer, P N New Methods for Preserving Bone, Skin and Blood Vessels *Postgraduate Medicine*, 12, 238-253 (Sept 1952)

*Kreuz, F P, Hyatt, G W, Turner, T C and Bassett, C A L The Preservation and Clinical Use of Freeze-Dried Bone *Journal of Bone and Joint Surgery*, 33, A 863 (Oct 1951)

nuclei, within the bone However, we have observed this staining characteristic in grafts which, while they have been in the host, had been non-viable for as long as two weeks We do not think that viable cells within the bone graft participate in the early phases of healing. Certain superficial cells may participate in the early phases but these, in our opinion, may not be significant.

MEDAWAR: I wish some clinician would express an opinion on Dr Longmire's classification into homostatic and homovital grafts I think it an excellent one—although not perhaps etymologically!

HYATT Into what classification would Dr Longmire put his fresh homogenous skin graft, if used as a life-saving procedure?

LONGMIRE If you're using it as a dressing, I think one might incorporate it in the homostatic group, but if one could use homografts of skin as most of us hope eventually to use them, as permanently surviving grafts, they would come in the homovital group

HYATT· You base the distinction on the fact that it has what apparently is a clinical take and stains and that, to all appearances that we have at our disposal, it grows? It doesn't fit in a category if it does not stain, is that it?

LONGMIRE The classification is based mainly on the end-result.

HYATT Functional

LONGMIRE I had not visualized the functional component as it was expressed today, but if you use that dividing line then the skin graft fits in the homostatic group

HYATT. Except that it is a bit confusing when you use the term homostatic and then demonstrate certain evidence of viability as we understand it.

LONGMIRE Not over a very long period of time.

MEDAWAR I should have thought that even the frozen-dried bone homograft was the source of some osteogenic stimulus, because there's a good deal of Swedish work on the osteogenic action of extracted bone. So evidently the bone homograft, even if it's frozen and dried, isn't merely a scaffolding, it is the source of some sort of inductive stimulus, even if it's non-specific But I gather that some of the Swedish work has not been confirmed, in the sense that even their extraction media, pure alcohol for example, have been found to be osteogenic

HYATT Electron microscopy has defined the structural unit of bone better than we have ever known it before*, to the point of placing the coarse fibrillar collagen, mucopolysaccharides, and the various metallic salts in their proper relationships We think, without definite proof, however, that the collagen and the mucopolysaccharides may be synthesized or resynthesized by the so-called osteoblast in the process of new bone formation We feel that the basic matrix of the homo- or heterograft of bone may not be as acceptable for such synthesis or re-synthesis In these points there may be a very valuable story.

*Robinson, Robert A , An Electron Microscopic Study of the Crystalline Inorganic Component of Bone and its relation to the Organic Matrix. *Journal of Bone and Joint Surgery*, 34 A, 389 (April 1952)

PARKES Is there any reason why a graft should not change its classification in the course of time?

LONGMIRE. I think that would add confusion to an attempt to clarify the situation

PARKES But if it starts by performing some vital function and the for some reason dies and remains as scaffolding?

LONGMIRE It seems to me that it is based more on what one eventuall expects of the graft If skin is used as a purely temporary covering tha you do not expect to survive, if it serves a purely mechanical function a was brought out today, it would be included in the one classificatio On the other hand, if you are expecting it to continue to grow an reproduce, it should be placed in the other category.

HUFNAGEL I would suggest that it be regarded, not as a classificatio but as a clarification of a concept In the first category, in which tl graft is of the framework type, structural integrity is of primai importance, but whether the transplant is living or dead is of litt consequence In the second group, in which it is necessary that certa cellular groups survive and maintain their secretory function (e endocrine transplants), cellular organization is of very little importan as long as there is adequate secretion of the hormone And then final there is the other category in which both structural integrity ar organization of the cells are important and the cells must also surviv If these factors are considered in this light I do not see how there can l any real confusion

A U. SMITH I felt that Dr Pate rather underestimated the possibil of frozen cells retaining viability The rate of cooling used by him is rapid, however, that the majority of living cells would not surviv

PATE· I was speaking primarily of the frozen grafts as they are in u in clinical surgery, and not as a laboratory procedure You can free grafts and get fully viable cells, but as it's done clinically in mc hospitals I don't think they are viable.

A U SMITH I'd like to hear of some of the hospitals who are freezi tissues in such a way that they would be viable

ROGERS Dr Jerome P Webster of the Presbyterian Hospital, w wrote one of the early papers on refrigeration of skin, stated that skin autograft, frozen rather crudely in an ordinary hospital refrigerat at 4°C and kept there for as long as three weeks, would survive a remain viable if transplanted back to the original donor before thi weeks had elapsed This is a clinical application of the freezing princi in which the grafts apparently survive despite the lack of any refi ments in the freezing techniques

PARKES I believe skin is very resistant to that kind of treatme Isn't that so, Prof Medawar?

MEDAWAR. Yes, very resistant indeed, but as Dr Billingham v explain in due course, skin does not survive being frozen *and* dried

PARKES Much depends on the method of reconstituting it In c experience of freeze-drying that may be even more important than t method of freezing It may be that if you haven't killed it by freezi and drying, you've killed it by your method of reconstitution

ROGERS: I heard Dr Cecil Taylor, of New York University, read a paper two weeks ago in which he demonstrated with his ingenious refrigeration chamber the fact that cells still maintain their viability even when frozen to extremely low temperatures, but these same cells are often destroyed by the reconstitution process.

PARKES You should reconstitute by putting back what you took out, which is presumably water and not saline

STRONG I wonder if anybody has done any work or knows of any work where diathermy has been used to thaw tissue from its frozen state prior to grafting. Would this possibly be a solution to some of our skin storage problems? Would there be greater or less survival if it was rapidly thawed by the use of diathermy? I know there is commercial equipment available that the Birdseye people have designed to thaw very large containers of frozen food very rapidly.

LOVELOCK I have made only a few abortive experiments with a diathermy apparatus, which I found did not deliver sufficient electrical energy to the frozen specimen to thaw it fast enough to be of any use. We are negotiating for a radar type of apparatus operating at a very high frequency and with fairly high power, which should provide (theoretically anyway) sufficient energy to do the job It certainly has worked on frozen solutions, but whether in practice it will work with tissues, it's a little early to say I think there is a possible future in that direction as you suggest

PARKES In the first test that Dr. Lovelock made, the material turned from a solid at $-70°$ into a vapour in a split second

POMERAT I think Dr Pate's paper is significantly exciting because we see that our goals may extend from complete viability of the tissues to a state where viability becomes actually undesirable Here is evidence then that viability in the artery appears to be undesirable and that we should seek a method of preservation which assures its physical properties, but with the elimination of antigenic bodies

EASTCOTT: This is a fundamental question we have been discussing, and I scent a severe cleavage in our ranks It would be valuable if we could have some indication that a homograft may survive It is generally accepted that the clinical use of corneal grafting is an example of a satisfactory homograft The evidence so far seems to me very slender that it is And with regard to the bone autograft, does it survive as a functioning group of tissue cells or not? We must know about this, because if these tissues work better as "cold meat", so to speak, then in trying to preserve life during storage we're going after something which is not going to help us in the end

RYCROFT: Before 1905 corneal graft surgery was almost entirely a surgery of heterografts, and the failures of the nineteenth century were put down in large measure to this cause But the two factors of infection and technique must also have had an influence Recently, it has been shown in London and in Geneva that under certain conditions heterografts between humans and cats and between cats and rabbits are possible, and these experiments may open up a new conception of corneal graft surgery From the clinical point of view, up to 1922

corneal homografts were mostly fresh, because of legal restrictions and the difficulty of obtaining suitable donors But in 1922, Filatov in Odessa published his work on the use of the cadaver cornea which he was able to obtain quickly after death (owing to facilities in that country) and to use within a few hours In this country we have not been able to obtain them legally at all until quite recently I think you will find most of the surgeons who do this work prefer the preserved cornea to the fresh material

STORAGE AND HOMOGRAFTING OF ENDOCRINE TISSUES

AUDREY U. SMITH and A S. PARKES

In this paper we shall refer only to viable grafts, ar
want to start with a very simple proposition, namely t
practical use is to be made of the technique of transpla
tissue, the surgeon must have suitable tissue available a
proper time It rarely happens that the right bit of t
becomes available spontaneously exactly when requ
and except in the case of skin, the scope for autografti
limited, so we are plunged immediately into problems
of storage in a viable state and of homografting of tissue.
The problem of homografting, the grafting of tissue
one individual to another of the same species, as Prof
Medawar has pointed out, is heavily tangled up with com
immunological considerations which apply with very diffe
force to different organs, different species and different
of transplantation. As a result, widely divergent views
found, based often on widely divergent experience We
already heard much in this Symposium of the doubts surg
feel and the difficulties they experience in the use of ho
grafts. On the other hand, many experimental endoc
logists, blissfully ignorant of immunological complicat
and with unlimited material at their disposal, have m
homografts of various tissues, notably mammalian gon
with complete nonchalance and notable effect Steinach
Lipschutz and many others, have grafted ovaries into m
mammals, testes into female mammals, and both into b
and have made observations on the resulting intersexu
without apparently being surprised that the grafts, essenti
homografts, took, persisted and functioned. Nor can it
supposed that such grafts necessarily broke down afte

short while. Lipschutz (1932), for instance, quotes in some detail the case of a castrated male guinea-pig which received an ovarian graft, became feminized, and for two years afterwards was used to demonstrate this condition The graft then turned androgenic, and in its third year after receiving the graft the guinea-pig with its male accessory organs in prime condition was used to demonstrate the potential androgenicity of the ovary. There are also many examples of the uterus, prostate and so on, having been grafted routinely to other individuals for various experimental purposes. We mention this point merely to emphasize the practicability and effectiveness of viable homografting in certain cases.

So far as the storage problem is concerned, a certain amount of experimental work has been carried out on the preservation *in vitro* of tissues by various methods, including cooling and drying No doubt we shall hear a good deal about this work in the course of this Symposium Of relevance to the present paper is the work of Lipschutz (Lipschutz and Uprus, 1927, Lipschutz, 1928) who was able to obtain active ovarian homografts in guinea-pigs from tissue which had been stored for many days, or even a week or two, at 14°C or between 1° and 3°C, as well as from tissue which had been partially desiccated. By contrast, tissue stored at temperatures below 0°C was invariably non-viable In general, it is fair to say that until two or three years ago comparatively little progress had been made with the problem of long-term preservation of normal mammalian tissue in viable condition.

We want now to give some indication of our work on these problems, and we must say immediately that in using the word *endocrine* tissues in the title we were thinking only of the ovaries, testes, adrenals and pituitary glands With the adrenal cortex our work has not reached a decisive stage, while that on the pituitary gland has been carried out, by our colleague Mr S E Smith, entirely by means of tissue culture In practice, therefore, our remarks will be restricted to the storage and homografting of gonadal, mainly ovarian, tissue

Grafts of Ovarian Tissue

Criteria of an active graft

In the case of the ovary it is comparatively easy to decide whether or not an active graft has been obtained, and we should emphasize that in the present paper the expression "active graft" means an endocrinologically active graft The gametogenic potentiality of the graft is an entirely different problem, which together with the general histology of gonadal grafts in the rat will be dealt with in the next paper by Dr. Deanesly.

In the rat, the criteria of an active ovarian graft are simple and relatively precise (see Harris and Eakin, 1949) If the ovaries of a rat are removed, the cyclic changes in vaginal epithelium, detectable by making smears of the vaginal contents, come to an abrupt end If, on the other hand, the ovaries are removed, cut into small pieces and implanted under the skin of the flank, the cyclic changes in the vagina start again after a short interruption. In other words, active grafts have been developed from the pieces of ovary This reaction is remarkably regular and remarkably rapid. In the strain of animals mainly used by us, the typical œstrous cornification of the vagina recurs in an average time of about seven days in almost all animals receiving an autoplastic graft of their own ovarian tissue Where the tissue used is less effective, either because of treatment of some kind or because it came from another individual, the proportion of animals in which active grafts develop is decreased and the average time taken for the graft to become effective is increased. This decreased effectiveness is presumably associated with destruction of a proportion of the cells of the tissue implanted. With the idea of trying to assess this loss we carried out a series of experiments in which decreasing amounts of fresh tissue were implanted The results (Table I) indicate that even when the amount of tissue implanted is reduced to one-quarter of an immature ovary, most animals develop an active graft in a comparatively short time, and they suggest that a high proportion of failures and a long latent interval indicate

Table I

EFFECT OF VARYING THE AMOUNT OF OVARIAN TISSUE GRAFTED (RATS)

Amount of ovary grafted	*No of animals*	*No of animals developing active grafts*	*Av time to restart of cycle (days)*
1/4 of one ovary	10	7	12 0
2/4 of one ovary	10	10	8 8
4/4 of one ovary	10	10	7 1
8/4 received two ovaries each cut into 4 pieces	10	10	6 2

that a very small amount of living tissue has been implanted or has survived the immunological reaction.

Against this background of information we can examine with some precision the effects of storage and homografting.

Effect of homografting

We have used two strains of rats (a) albinos, derived (a long time ago) from Wistar stock, and (b) hooded, from a colony maintained at the Institute for many years Preliminary experiments showed that the albinos were less receptive of homografts, whether inter- or intra-strain (Parkes and Smith, 1958) and almost all our work has been carried out with the latter strain Further information is given in Table II, arranged according to the percentage of animals developing active grafts The average time taken to do so varied between 8 4 and 12 3 days, and was thus above that for autografts It may be concluded that homografts have a somewhat reduced

Table II

HOMOGRAFTING OF FRESH OVARIAN TISSUE IN ALBINO AND HOODED RATS

Strain	*Received grafts from*	*No of animals*	*Animals developing active grafts*	
			Number	*Per cent*
Hooded	Hooded	10	9	90
Hooded	Albino	19	16	84
Albino	Hooded	18	11	61
Albino	Albino	10	6	60

chance of becoming established in these strains of rats, and take rather longer to do so. It should be emphasized, however, that perfectly effective homografts can readily be obtained. The ultimate fate of such homografts by comparison with that of autografts has not yet been determined, but they are known to last for at least several months.

Storage above 0°C.

In early experiments we were able to obtain a number of active grafts from ovarian tissue removed from the animal and stored in damp cotton wool for some days at about 2°C.,

Table III

EFFECT OF KEEPING DEAD RATS AT +2°C BEFORE REMOVING OVARIAN TISSUE FOR SOAKING IN 15 PER CENT GLYCEROL-SALINE AND GRAFTING, WITH OR WITHOUT FREEZING FOR ONE HOUR AT −79°C

Time body at +2°C (days)	*Treatment*	*No of animals*	*No of animals developing active grafts*	*Av time to restart of cycle (days)*
1	Soaked only	10	0	10·8
1	Soaked and frozen to −79°C	10	4	17·8
2	Soaked only	10	7	12·1
2	Soaked and frozen to −79°C	10	2	20·0

thus confirming Lipschutz's observations. However, it soon became evident that if isolated ovarian tissue could be obtained it could be preserved *in vitro* much more efficiently than at 2°C, and attention was then turned to the question of how long ovarian tissue would remain viable in a dead body. Experiments were carried out in which rats were killed by a blow on the head and put in cold store at +2°C.; one or two days later the bodies were opened, the ovaries removed and used for grafting experiments. The results (first and third lines of Table III) show that one day after death the viability of the ovarian tissue is slightly, and at two days substantially, impaired. It must be remembered, however, that these results

relate to homografting of tissue cut up and soaked in 15 per cent glycerol-saline. In view of these additional hazards the effect of the staleness of the tissue cannot have been very great. It appears, therefore, that freshness of tissue intended for grafting is not so important as is sometimes supposed.

Storage below 0°C.

The main results we have to bring before you are themselves somewhat stale by now, but they are very relevant to the theme of this Symposium. About four years ago we (Polge, Smith and Parkes, 1949) made the discovery that glycerol has remarkable properties in protecting living cells against the otherwise fatal effects of freezing and thawing. This discovery was of considerable interest because it has usually been assumed that if living cells could be frozen and thawed without damage they could be kept indefinitely at temperatures low enough to arrest metabolic activity, e.g. those obtained by the use of solid carbon dioxide (—79°C.) or liquid air (—190°C). It now appears that physical effects may come into the storage picture and that the nature of the medium may be very important, but substantially the assumption was probably correct.

The use of glycerol was rapidly applied to certain other living cells and tissues, notably ovarian tissue, and the possibility of indefinite storage of tissue *in vitro* was opened up We now have a large amount of information about the preservation of ovarian tissue The first step was to investigate a number of different media containing glycerol in saline or serum, together with various conditions of freezing. It was found that optimal preservation was obtained by slow freezing to —190°C. in 15 per cent glycerol-saline. A suitable freezing gradient is obtained in the special type of slow cooler (Polge and Lovelock, 1952) shown in Fig 1 Results with different media are given in Table IV —79°C was found not to be a satisfactory temperature for storing ovarian tissue (even for comparatively short periods) frozen in glycerol-containing media The solitary promising result obtained

after nine days at −79°C. in this series of experiments was with tissue frozen in neat rat serum, but the time taken to establish an active graft was very long. It is noteworthy that grafts made from tissue frozen under the best conditions

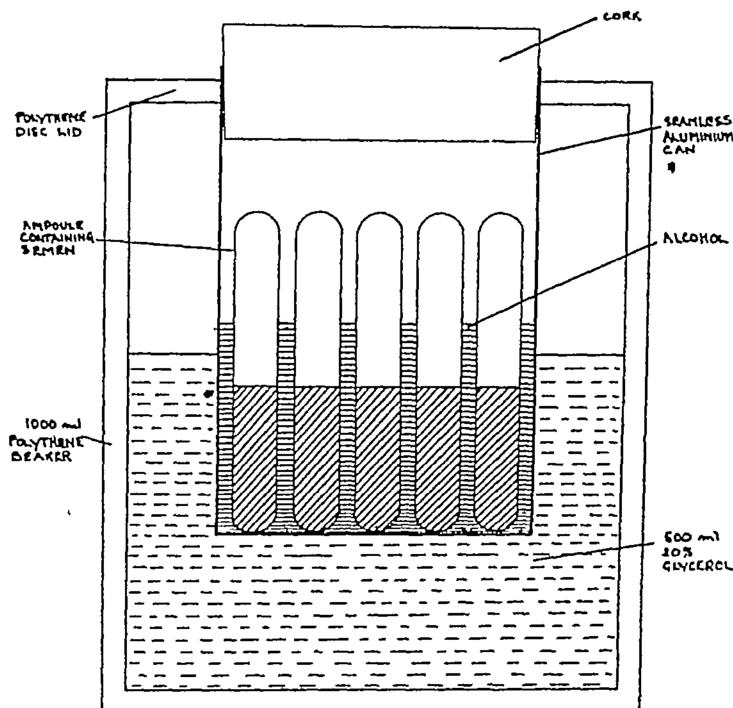


FIG 1 The construction of the cooling vessel
Outer vessel 1,000 ml polythene beaker Height, 18 0 cm, diameter, 12 0 cm, thickness, 0 5 cm, charged with 500 ml of 20 per cent glycerol
Inner vessel Aluminium seamless can Height, 10 0 cm, diameter, 7 0 cm, thickness, 0 025 cm
(Polge and Lovelock, *Vet. Rec.*, 64, 396, 1952)

so far ascertained had an average latent interval much greater than for grafts made from untreated tissue, so that if the data given in Table I apply, there must have been some considerable loss of cells on freezing. It is possible, however, that some other factor than mere quantity of living cells is involved, because frozen and thawed tissue

Table IV

RESULTS OF GRAFTING OVARIAN TISSUE FROZEN FOR NINE DAYS IN VARIOUS MEDIA, IN COMPARISON WITH CONTROLS

Treatment of ovarian tissue	*Proportion of animals showing active graft within 28 days*	*Average time to establish active graft (days)*
Unfrozen control 15 per cent glycerol-saline at room temperature for one hour	14/14	6 9
Frozen in 15 per cent glycerol-saline to −196°C	5/5	14 4
Frozen in 15 per cent glycerol-serum to −196°C	5/5	16 4
Frozen in 25 per cent glycerol-saline to −196°C	3/5	23 8
Frozen in Serum to −79°C	4/4	24 0

(Taken from Smith and Parkes, 1951)

seems to have lost much of the capacity of fresh tissue to survive *in vitro* at ordinary temperatures (Parkes and Smith, 1953), and may, therefore, have suffered some qualitative change

The next step was to investigate the effect of prolonged storage at low temperature, using the optimal conditions of freezing so far evolved. The results are shown in Table V,

Table V

RETENTION OF VIABILITY IN LONG-FROZEN OVARIAN TISSUE (−190°C)

Duration of freezing of tissue (days)	*No of implantations*	*No of active grafts obtained*	*Average time taken for re-appearance of cycle (days)*
1/24	5	5	15 4
9	5	5	14 4
83	5	5	18 8
117	8	8	15 1
285	9	9	16 0
367	12	12	16 3

and are of great interest in showing that the damage caused by freezing and thawing, as assessed by our two criteria, is not increased by prolonging the period of storage at −190°C. from one hour to one year. We have adequate supplies of this material in store and shall continue to test it at intervals, by autografting, until the donors have perished of old age, after which homografting will have to be resorted to So far as we can tell at present, however, rat ovarian tissue is potentially immortal under the conditions described Unfortunately, the experimental investigation of potential immortality cannot be expedited very much, and it will be a long time before we know the ultimate fate of tissue so stored.

All the experiments described above on storage below +2°C. involved the autografting of tissue frozen while fresh. The slight loss of effectiveness caused by freezing and thawing is cumulative with that caused by homografting (Parkes and Smith, 1953). We have now shown further that the additional hazard of the tissue not being fresh further decreases the viability of frozen homografts, but even so, active grafts can be obtained from tissue taken from a two-day corpse, frozen, thawed, and implanted into another individual (Table III).

Other Tissues

Results essentially similar, but less extensive, have been obtained with the storage and homografting of testicular tissue taken from infantile rats Such tissue implanted into castrated adult males readily forms homografts, the activity of which can easily be judged by the mating behaviour of the animals, and by the size of their accessory organs The capacity of the testicular tissue to make endocrinologically active grafts in a test of this kind is decreased only slightly by freezing in 15 per cent glycerol-saline and by prolonged storage at −190°C Unlike the ovary, the rat testis can be stored with considerable success at −79°C in 15 per cent glycerol-saline Tissue left in the body at +2°C. for one or

two days retains some power to make an effective graft, but as with the ovary, stale testis freezes less well than fresh testis

Results with adrenal cortical tissue are so far less promising, but some slight success has been achieved in maintaining adrenalectomized animals on a salt-free diet by means of a graft derived from tissue which had been frozen in 15 per cent glycerol, thawed, and implanted subcutaneously At present we know nothing about the prospects of long-term storage of this tissue.

In the course of the work on the gonads we have made incidental observations which show that parts of the reproductive tract, particularly Fallopian tube and epididymis, readily make grafts after freezing and storage at low temperature

The experiments summarized in Tables I, II, III and V have not been recorded previously, and we have again to acknowledge the assistance of Miss J Mason and Miss M Mansi who carried out the surgical procedures involved as well as making the day to day observations.

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[Discussion of this paper was postponed until after the paper by Deanesly which follows (See p 93)]

HISTOLOGICAL EVOLUTION OF RAT GONADAL TISSUE TRANSPLANTED AFTER FREEZING AND THAWING

RUTH DEANESLY

Ovary Grafts

Introduction and technique

THE autografting and homografting of normal rat ovaries have been studied in connection with different problems by a number of experimental workers. Among these are Goodman (1934), Pfeiffer (1934) and Biskind and Biskind (1944) who investigated the effects of the host's secretions on the grafts, and Harris and Eakin (1949) who published a comprehensive account of the reactions of autografts, various kinds of homografts and heterografts in normal and ovariectomized rats

Recently, Parkes and Smith (1952) have described the growth of rat ovarian tissue grafted after freezing and thawing This work was done on 60 to 100 g rats whose ovaries were chopped into small fragments about 1 mm.^{3} It was hoped that a fuller histological study of the effect of freezing on graft development would be possible if immature ovaries from rats one week old were used and cut in only two pieces before they were treated according to the same technique. Half the implants were soaked for one to two hours in 15 per cent glycerol-saline, and formed a control group, while the others similarly soaked in glycerol-saline were then gradually frozen and kept for one hour at −79°C They were thawed immediately before being placed just under the skin in ovariectomized 100 g. hosts.

Grafts were fixed one, two, three, four, six, eight and ten days after implantation, and sections were cut serially. To judge from the numerous early stages which were examined,

the progress of a graft depended largely on local conditions, and the speed with which it became vascularized.

Histological changes in the grafted ovaries

In Parkes and Smith's material in which the pieces of grafted ovary were from older rats all follicles including primordial follicles disappeared after freezing and grafting and were apparently reformed between the third and seventh days

In the present series, in spite of the mass destruction of follicles and primordial follicles, a certain number survived both soaking in 15 per cent glycerol-saline and freezing to −79°C These surviving follicles continue to grow, but how many may approach normal œstrous size in older grafts cannot be estimated It is well known that follicles at all stages of growth are readily damaged and in a normal ovary a proportion of follicles are constantly ceasing to mature and undergoing degenerative changes throughout the life history

The ovary of the seven-day rat is packed with follicles and primordial ova Follicles of the largest group (diameter 80–85 μ) have three to four rows of granulosa cells and no antrum. After soaking in glycerol-saline most of the follicles show severe damage, but some primordial follicles survive and also some of the granulosa cells in the larger follicles After soaking in glycerol-saline and freezing to −79°C. the damage is rather more severe, affecting a higher proportion of the follicles, but the two types of treated ovary do not appear very different in section

One day after grafting, a control ovary shows large degenerating areas infiltrated by leucocytes, but the graft is well vascularized and normal ova and follicles can be found

At this stage two similar frozen grafts from the same animal may differ markedly in histological appearance. A highly vascularized one-day frozen graft shows invasion by collagenous connective tissue, but contains some conspicuous follicles with one to two layers of epithelium which appear to

have survived with their eggs undamaged, and also some primordial follicles. The larger degenerated follicles and eggs are disappearing rapidly. A graft in the same section shows numerous degenerating follicles, but apparently no good eggs. It is infiltrated by leucocytes, as is the frozen graft of the same age from another rat This graft, however, includes 45–50 μ diameter follicles with one to two layers of epithelium apparently undamaged and occasional healthy looking primordial follicles. These healthy survivors twenty-four hours after freezing and grafting form a very small proportion, perhaps 1 to 5 per cent, of the original total (primordial follicles included) present in the graft Follicle loss in untreated grafted ovaries has not been studied, but some control grafts soaked in glycerol-saline show almost as much damage to follicles as the frozen ones.

Material was available from two rats showing the condition of frozen ovaries two days after grafting. In both animals the tissue showed a remarkable reorganization and included healthy follicles up to 70 μ diameter In one, most of the damaged tissue seen at an earlier stage had disappeared, but in the other some persisted in the middle of the implant. Not all the granulosa cells of the larger follicles which had lost their eggs had been adversely affected; round masses of them could still be seen, apparently healthy but irregularly arranged.

Comparing a control implant, also grafted two days, we find less degenerate tissue and follicles slightly larger (up to 85 μ diameter) and more numerous, but it cannot be said that it is essentially different from the frozen graft of the same age

At three days a frozen graft shows primordial follicles of different sizes and some degenerate tissue, and at four days it contains some follicles up to 130 μ diameter with three to four layers of epithelium and some primordial follicles at the edge.

In a four-day control graft there are various sized follicles, including one slightly larger than any in the frozen graft.

Another has healthy two- and three-layered small follicles and primordial follicles and little groups of granulosa cells from which the egg has disappeared

After six and eight days, control and frozen grafts both show larger follicles, 150–200 μ diameter. The largest of these have a thickened theca interna—an indication that premature luteinization is likely. Small groups of granulosa cells —anovular follicles—are common. At ten days after grafting some of these small groups of cells appear luteinized Up to this stage there is no luteinization of the larger follicles. Anovular follicles—organized groups of granulosa cells—are not uncommon in the older grafts, and some of these may be new formations, as described by Parkes and Smith, while others derive from follicles whose egg has not survived.

The remainder of the experimental animals were killed at twenty-eight and twenty-nine days, when they showed œstrous cycles and were found to contain healthy grafts which resembled those described by Parkes and Smith. They were well grown and often had several series of corpora lutea, though true ovulation does not usually occur.

A typical frozen four-week graft contains older corpora lutea, some with the remains of the egg still visible in the centre, recently luteinizing follicles and smaller follicles, one with an apparently good egg Another frozen graft of the same age includes Fallopian tube, corpora lutea of two generations and a large follicle (820 μ diameter) with swollen theca interna which is about to become luteinized. Grafts similar to this in all respects are found in the unfrozen series

The excessive theca development common in ovarian grafts is attributed by Biskind to pituitary gonadotrophic secretions unaffected by œstrogens.

It may be concluded from this series that freezing the immature seven-day ovary to −79°C does not affect its reaction to grafting as compared with that of the unfrozen glycerol-soaked control.

Testis Grafts

Introduction and technique

Homografting of testis has been carried out for many years with greatly varying results. Moore and his collaborators, and Lipschutz, have done much to correct the errors of Steinach and earlier workers who held that the germinal epithelium necessarily became degenerate in grafts though the interstitial cells proliferated. In 1924, Moore reported active mitosis in the germinal epithelium of guinea-pig testis grafts, but up to that time he believed that no one had found spermatozoa in a mammalian testis graft apart from a single instance recorded in this particular paper where an immature testis was grafted into the scrotal sac of a castrated sixty-day rat and examined after six months Later, Moore reported further cases of spermatozoa in grafts, but only in scrotal grafts Some workers have attributed absence of full spermatogenesis to absence of excurrent ducts, but Moore's findings do not support this view (Moore, 1939).

Turner (1938) described a comprehensive series of homotransplants of prepuberal testes into normal and castrate male and female rats He experimented with different body sites but obtained his best grafts from within the anterior chamber of the eye. Some of the grafts contained sperm heads thirty-eight days after transplantation. In general the grafts showed hypertrophy of the interstitial cells, and these maintained secretory activity in seminal vesicles and prostate in castrate hosts. Turner attributes the full spermatogenesis in some grafts to their early vascularization and to the lower temperature of the eye chamber, which approximated to that of the scrotum and was 4° to 6°C. below that of other body sites. The present experiments were begun by A S Parkes to study the reactions of testis tissue to freezing and thawing. Immature testes from rats about seven days old were cut in two and after treatment placed subcutaneously in the flank of castrate rats like the ovaries already described. Such grafts soaked in 15 per cent glycerol-saline or soaked, gradually frozen to −79° or to −190°C for varying periods and then

thawed, "took" readily and caused growth and secretion of the seminal vesicles of the hosts, many of which mated with normal females. After four to sixteen weeks the grafts varied histologically, but to about the same extent in frozen and unfrozen material In general the tubules were small, often with only a single row of germinal epithelium and Sertoli cells, but sometimes with one or more layers of primary spermatocytes In the lumen of the tubules could be found occasional multinucleate giant cells and cell debris Interstitial cells were well developed and seminal vesicles up to full normal size occurred in rats with successful grafts from both frozen and unfrozen tissue

Early stages and later growth

In further experiments a study was made of the early stages and later growth of both sets of grafts. At the stage used for implantation, sections through a control glycerol-soaked testis show tubules 50–60 μ diameter, lined by a single layer of spermatogonia and Sertoli cells, with a few cells free in the lumen. Giant spermatogonia are fairly common, with nuclei from 12–20 μ diameter, some in mitosis. The interstitial cells are small and after fixation form only a thin network in the inter-tubular spaces.

A similar testis, fixed after freezing to —79°C for seven days and thawing, is histologically indistinguishable. No cells are obviously damaged, and giant spermatogonia in mitosis can still be found After three days the half testis implants, both frozen and control, show considerable areas of degenerating tissue but the outer tubules are healthy and have already begun to enlarge—to diameters of about 70 μ in the frozen graft and up to 95 μ in the control Both grafts contain mitoses in the tubules and the interstitial tissue. Leucocytes are fairly common in the intertubular spaces but only abundant near the decaying tissue After six and eight days grafting the latter has been further absorbed and both types of graft show continued development, tubule diameters reaching 100 μ but showing considerable variation. On a

direct comparison of tubule size and general appearance it is seen that the control grafts have developed rather further in the same time than the frozen ones, but the difference is not great, some of the eight-day frozen group corresponding to six-day control grafts

At fourteen days, increased mitotic activity can be found in the germinal epithelium in both sets of grafts, some tubules containing two or three generations of primary spermatocytes and many mitotic figures, while the majority are still in the resting state. Interstitial tissue is relatively abundant, but is infiltrated with leucocytes. Some of the tubules contain cell debris Up to about twenty-eight days the average tubule diameter in the grafts (examined by direct comparison of successive stages) continues to increase though there is much variation both in size and degree of differentiation in the same section Diameters range from 135 to 230 μ in twenty-eight day frozen and control grafts, as compared with 220 to 280 μ in similarly fixed normal testis. Germ cell differentiation in the twenty-eight-day grafts does not go beyond the secondary spermatocyte stage. Thereafter, cells degenerate and pass down into the *tubuli recti* and *rete testis* where this is incorporated with the graft Some tubules contain few or no spermatogonia, only a lining of typical fringed Sertoli cells.

Other experiments showed that prepuberal testis tissue frozen at —190°C. for seven weeks or longer would still grow as a graft and prove functional. After eighty-seven days the seminal vesicles of a castrate host weighed 0·7 g The interstitial tissue was well developed in this graft but the tubules showed no advance in differentiation or size as compared with those of the twenty-eight-day frozen graft. At most there were three or four rows of spermatocytes with degenerating cells in the lumen

The next series of soaked and frozen testis grafts was implanted in the scrotum each castrate host received one prepuberal testis cut in four pieces Grafts were fixed and examined histologically at weekly intervals for five weeks. They did not "take" quite so well as the subcutaneous ones,

probably owing to vascularization difficulties At thirty-five days some tubules in both groups showed active mitosis of the germinal epithelium, but no spermatids or spermatozoa were found Twelve weeks after grafting, however, four rats, one with control and three with frozen grafts, showed full spermatogenesis The spermatozoa were motile and the active tubules histologically normal. Other tubules in the same section had lost their shape and looked completely degenerate. Interstitial cells were well developed and seminal vesicles large

These experiments show that prepuberal testis tissue may be frozen to —79°C, and after thawing may resume full differentiation and under favourable circumstances produce normal spermatozoa.

Summary

Prepuberal rat ovaries and testes retain their capacity to grow and differentiate as homografts after freezing to —79°C. and gradual thawing

The operations referred to in this paper were carried out by Miss J. Masson and Miss M Mansi

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DISCUSSION

ZUCKERMAN I should like to refer to some experiments which we are carrying out, in collaboration with Dr. Parkes and Dr Smith, in order to see whether frozen ovarian autografts behave differently from unfrozen autografts Littermate controls have been used, frozen tissue being implanted into one sib, and unfrozen tissue into another In

general, the results appear to confirm what Dr Deanesly has said There is no particular difference between the two types of grafts, but the frozen graft appears to take a little longer time to emerge from the phase of degeneration We have been working with animals much older than the two-week-old creatures to which Dr Deanesly referred in her communication—our animals were all about one hundred and fifty days old—and it seems that even in these animals there is some persistence of oocytes at the end of the thirty-day period.

I should like to comment on the possibility that oöcytes are regenerating in these tissues My own view is that the evidence does not favour such a conclusion First of all, one does not see the nuclear changes indicative of oögenesis I think the slides Dr. Deanesly has shown make this quite plain Oocytes seen in grafts of mature tissue are in the resting stage of the pro-phase We do not see cells actively dividing, as, for example, in the seminiferous tubules or in the ovary of the newborn rat. [Here two slides were shown to illustrate oögenesis in the young rat]. As far as I can make out, the oocytes seen in grafts of mature rat ovary represent the persistence of oocytes that are present in the tissue when grafted The view that neogenesis of oöcytes does not occur in such tissue agrees with what we know about the normal process of oogenesis in the rat Furthermore, the rate at which oöcytes disappear from grafted tissue varies inversely with the amount of ovarian tissue that is present

GAILLARD Dr Stolk from my department has done a series of the same kind of experiments* as Dr Deanesly, only with non-frozen tissues, and practically all his pictures are just like hers We have the best results if the peri-ovarian spaces develop after grafting Those little spaces seem to me to be very necessary for good development of an ovarian graft Did you ever see such a space develop?

DEANESLY: In early grafts

GAILLARD Fifty-seven days after grafting, when the little ovarian structures have developed, they have their own extra-cœlomic cavity

MOLLISON Was glycerol removed from those tissues when they were grafted?

PARKES No, the preparation was just thawed out, and the pieces of tissue popped in—in fact probably quite a lot of medium as well

MOLLISON Have you any idea how much glycerol got into the cells?

PARKES They were soaked for one hour before freezing, and I imagine glycerol got in fairly well Is that right, Dr. Lovelock?

LOVELOCK I think so

MOLLISON What would be the proportion by volume of glycerol to tissue?

PARKES Very large

MOLLISON Gross excess in solution?

PARKES Yes Not necessarily when implanted, but certainly when frozen.

PATT: What technique of thawing did you use?

*Thesis, State University of Leiden, 1951.

PARKES Plunging ampoules into a water bath at +40°.

EICHWALD You spoke about the potential immortality of your ovarian grafts, and as the criterion of this immortality you took the changes in the vaginal epithelium I wonder what is immortal? Are the cells which you transfer, or is it the hormone which has been formed in the cells before they were removed from the donor?

PARKES When I spoke of immortality, I was of course referring to the tissue in its frozen state, the viability of which can be demonstrated by thawing and grafting at any time after you've frozen it The collection of hormone in the tissue might have some very small effect, but once the cycle has started again it goes on for quite a long time The cycle is not necessarily very regular, and many of the mice finish up with permanent, persistent cornification, but there's no reasonable doubt that the cells of the graft are forming new œstrogen.

DEANESLY In a six-day graft the fallopian tube, which must be very immature when it is put in as a graft, is markedly stimulated, so that even at six days there's a certain amount of œstrogen being produced

ZUCKERMAN Surely there are no circumstances in which a graft will continue functioning in this way for ever? How long do you believe it continues producing hormone?

PARKES From our own work we only know that such a graft functions for some months, but I quoted the example of Lipschütz's guinea-pigs to show that there are historical records of homografts functioning for a very long time

KROHN On the basis of the work with skin homografts one might expect that ovarian homografts would all be destroyed, provided that there was sufficient genetic dissimilarity between host and donor If, perhaps, you get only a proportion of successful grafts it would be interesting to see whether those animals which accept ovarian tissue will also accept skin from the same donor and whether those animals which reject the ovarian tissue also reject skin

BILLINGHAM Dr Parkes and I are carrying out experiments to determine the extent to which skin homografts can be exchanged successfully between female rats from his stocks and, more particularly, to determine whether the reactions of these rats towards skin homografts parallel their reactions to ovarian homotransplants Unfortunately the work hasn't progressed far enough to enable me to answer Dr Krohn's questions

MERRILL Have any observations been made on the difference between the intervals of starting the cycle or perhaps the persistence of the graft or its activity, when the recipient is an adult animal or when it's an immature animal?

A U. SMITH Grafts have been made into adult animals and into animals which are only just reaching maturity and they seem to do equally well in both The age of the host seems to make much less difference than the age of the donor ovary

PARKES When a very immature ovary is transplanted into an adult host, as shown many years ago, the graft matures at a great rate, but we haven't used any hosts which have been very immature

ROGERS. A year or two ago I received a report from Dr. James Hamilton, an anatomist in New York, of some interesting glandular transplantations which had been performed in a large mid-western State Penitentiary In this State, sex offenders are castrated, and a doctor working at the penitentiary where these offenders serve jail terms, used minced, testicular tissue as homografts, transplanting it into their buttocks The homografted tissue apparently survived, and helped to restore some secondary sexual characteristics This work has not been reported as yet, but I think it is a very interesting finding.

KROHN· It really depends on how long the graft was active. I should have thought that most probably it served only as an implant of hormone

ROGERS I don't know how long their follow-up period is. Apparently the work has been in progress for about two years now

PARKES This is a subject which has to be handled with some discretion. Many of you may remember that twenty-five years ago the whole subject got into some disrepute owing to the efforts of Voronoff, with his monkey-glands and so on That was heterografting on a grand scale and I think put the study of this matter back a good many years

DEMPSTER But you shouldn't come out against Voronoff if you use the same technique of pushing a little bit of something underneath the skin

PARKES The essential difference was the heterografting from one species to another as compared with homografting.

DEMPSTER But it's the whole technique that I really object to.

PARKES That's another matter. Let's hear about your objections.

DEMPSTER What sort of connective tissue cells were you getting in these homografts? Weren't there some lymphocytes in your sections? I presume these homografts were destroyed at some time

DEANESLY. Why do you presume they were destroyed?

DEMPSTER· Well, it's generally the case, except of course that the rat is such a convenient animal But usually homografts are destroyed at some period in that animal.

DEANESLY· But the testis homograft has the benefit of the host's anterior pituitary secretion and it grows so actively that it appears to be able to laugh at immunological reactions You could see the development of the cells and the steady growth

DEMPSTER Was there no evidence of the connective tissue cells of the testis showing any curious reaction?

DEANESLY There was rather more of it proportionally because the tubules were not so well developed as in the normal testis

DEMPSTER· The ovary of the dog can be transplanted with an immediate blood supply this is effected by transplanting the ovary with its adjacent kidney from whose artery the ovary derives a blood supply. The homotransplanted kidney stops secreting after a few days in the dog At the moment I am studying the changes in the kidney, adrenal and ovary (all in one block of tissue) at the fourth day after operation at a time when the kidney is still secreting Each organ shows the same changes occurring in the endothelial cells of the capillaries and

arterioles and in the connective tissue cells The endothelial cells swell up and become pyronin-positive, the connective tissue cells swell up, become Fagræus cells and then mature plasma cells. In spite of these changes the parenchyma of these organs appears to be normal I interpret this pyroninophilia as being a reaction of the organ against the host

KROHN I think Dr Deanesly made a very important remark when she said that the presence of the anterior lobe of the pituitary enabled a gonadal graft to laugh at the immunity reactions That seems to be the whole difference between endocrine tissues and other tissues like skin which have no trophic hormone from the pituitary to stimulate their growth I wondered whether anybody had put grafts of gonadal tissue into hypophysectomized animals to see whether, in the absence of trophic stimuli, they suffered the fate of skin grafts

PARKES It is perfectly true that a graft will not take readily if the two existing ovaries or two testes are left in position to mop up the supply of gonadotrophin Can anybody answer that question about the hypophysectomized anumals?

EICHWALD I believe Dr Woodruff has done that, and Dr Dameron in St Louis, and whenever that was done the trophic effect was gone

MERRILL With regard to the kidney, I'd like to raise another point along this line It is true that when one removes a single kidney from a normal animal the other kidney will hypertrophy, and whether that is simply due to a "work load" (which is a word that has come into some disrepute in renal function) or whether it's due to a true trophic influence, perhaps from the anterior pituitary, we don't know However, you can get similar changes in an animal who is in a static situation, as far as the work load goes, by the injection of growth hormone I wonder if the observation has been made upon renal homografts (perhaps Dr Dempster might know?) with regard to the grafting of a homografted kidney into an animal which has been bilaterally nephrectomized, with and without pituitary, or with and without growth hormone

DEMPSTER A homograft doesn't last long enough The hypertrophy after unilateral nephrectomy takes several months, doesn't it? Or are we talking about rats again?

MERRILL I don't know about the dog In the hypophysectomized rat the growth hormone effect is noticeable within four to five days, although there is some question as to whether the renotrophic effect is the same as the general somatotrophic effect In the partially nephrectomized animal, significant increases are found in less than three weeks, the amount depending on the diet, among other things

DEMPSTER I can't argue from the rat, but certainly in the human the blood urea rises, and according to some authorities the blood urea is not really back to normal for about nine to ten months And in dogs, if you remove one kidney the blood urea rises a certain amount If you bilaterally nephrectomize a dog and transplant one of the kidneys either to the neck or to the iliac vessels its weight will increase over a year from say 60-70 g when you transplant it, to about 110 g when you remove it about a year later

MERRILL Do you know of any observations in which the survival of a homograft performed under these circumstances has been compared with homografts under other conditions?

DEMPSTER All homografts and autografts do swell up immediately after you've transplanted them and it's quite a problem to work out the actual rate of swelling of the homograft Certainly within seventy-two hours a homotransplanted kidney increases its weight threefold although the renal circulation is still quite normal, the interstitial cell reaction described previously is going on and the kidney is producing urine

PARKES I think we should be the first to agree that any conclusions drawn from the ovaries and testes should be applied to other organs with great caution But the fundamental fact remains that much of our basic knowledge of the gonadal hormones was obtained by heterosexual grafting years ago, and those seem to me to be essentially homografts

ZUCKERMAN You said that of the two strains of rats used, the hooded rat behaved better than the albino Now many workers, and we amongst them, have the experience that ovarian homografts do not take very well In two separate studies we have failed to establish ovarian homografts in adult rats

DEANESLY Were they castrates?

ZUCKERMAN Castrates and normals Furthermore, if we make an autograft in the monkey—just remove the ovary from its normal position and put it somewhere else in the body—we find that the ovary decreases in size.

I should like to know to what you attribute the difference between the two strains of rats

PARKES: It's just an unfortunate phenomenon that we can't explain.

ZUCKERMAN You also referred to the pituitary We have tried very hard to repeat observations that have been reported about pituitary transplants, but we do not find that homografts take In ferrets we find that at most 15 per cent of the original tissue survives at the end of about six months

PARKES Do autografts of pituitary take?

ZUCKERMAN We believe so If you divide the pituitary stalk, leaving the pituitary *in situ* or, as Harris described, remove the pituitary and replace it in the sella turcica, the pituitary is not affected at all

MEDAWAR I think it is very misleading from a genetical point of view to talk about autografts and homografts as if they were qualitatively distinct What one is dealing with is various *degrees* of genetical affinity and there is a spectrum of such affinities, beginning with the autografts at one end (where you have genetical identity); then there is the relationship between highly inbred animals, where perhaps there is not quite such a high degree of affinity, and the spectrum goes all the way to the other extreme, where there is gross genetical disparity between donors and recipients For example, in Lipschütz's grafts which you have referred to, we don't know anything about the genetical background of his guinea-pigs—he may have been dealing with a highly inbred stock, in which case he was dealing more or less

with autografts or with grafts having a comparable degree of genetic similarity.

A. U SMITH But there is Whitney's work on transplanting foxhound ovaries into Dalmatians There could have been very little genetic relation between those strains, but the ovaries functioned very well He homografted into the ovarian capsule and the recipients subsequently produced litters

MEDAWAR I'm not denying that these homografts or autografts work, I'm merely making a terminological point which I think we ought to bear in mind

PARKES A perfectly sound point

DARCY When you reported a percentage of failures in your ovarian homografts (the smallest dose, the one-quarter ovary), were these simply endocrinological failures, or were they also histological failures?

PARKES We do not know I don't think we've ever had a case where we found a physically good-looking graft which was not functioning endocrinologically

DARCY But it's curious that with the smallest dose you did have failures

PARKES Yes But we may not have allowed a long enough time Vaginal smears were continued for only about twenty-eight days.

ROGERS I hope we aren't going to prevent clinicians from transplanting tissue homografts by raising the old fear that they will be classed with men like Voronoff If you have a case of a young boy who is a bilateral cryptorchid eunuch you want to give that boy secondary sexual characteristics Of course, we do have hormone therapy today which will help the boy, but if we could put homografts of minced testicular tissue into this child and carry him along permanently with actively secreting hormonal tissue, I think we have a better solution to the problem

PARKES I should have thought that was well within the discretion of the clinician

TRANSPLANTATION OF CULTIVATED PARATHYROID GLAND TISSUE IN MAN

P J. GAILLARD

USING methods for the cultivation of tissues from animals of different species, strains of growing cells can be obtained. Usually, however, after prolonged cultivation many of the histiotypical and organotypical characteristics are lost and more or less pure strains of either spindle-shaped or epithelial cells are obtained, depending on the original tissue and on the *composition of the media used.*

Earle has shown that pure strains of, for example, spindle-shaped cells can be obtained from one cell only and that even in such cases great variability in the shape of the cells is regularly found. No specific morphological differences can be observed on comparing "fibroblastic" strains derived from different parts of the organism, but this does not necessarily mean that no functional differences exist. It could be demonstrated that, for instance, three different strains of the fibroblastic type derived from different parts of the same chick embryo react quite specifically if cultivated in a comparable series of different concentrations of a growth-promoting "substance" (Gaillard, 1931, 1935, 1942) Nevertheless it remained most intriguing that the specific morphological characteristics were lost after prolonged cultivation, and therefore a closer analysis of this special point was attempted (Gaillard, 1931, 1935, 1942)

In this connection it was considered to be of major importance that during embryogenesis, and also in the period following birth, a continuous change in the composition of the "body fluids" is likely to occur and accordingly one might expect these "changes" to be of importance for the realization of cellular and tissue differentiation phenomena

It stands to reason that the general humoral milieu is not the only important factor in keeping the morphological structure of explanted tissues intact, but with a number of tissues it could indeed be demonstrated that the occurrence of histological differentiation processes greatly depended on the "age" of the donors from which the media were prepared.

It was found that complete osteogenesis occurred in a "pure strain" of periosteal fibroblasts after cultivating them successively in a series of press juices prepared from embryos of increasing age (Gaillard, 1931, 1935).

Verdam (1946) described how explanted cartilagenous primordia of long bones from 15–30 mm. rat embryos showed progressive differentiation only if press juices from younger embryos were completely omitted.

In the same way, explants of the anterior hypophysis from a young rabbit retained organotypical morphological characteristics for a period of two to three weeks if great care was taken not to use "body fluids" derived from embryos at all (Gaillard, 1937, 1942).

As a consequence of these results it now seemed possible in principle to culture organ fragments without disturbing organotypical characteristics within some weeks of explanting them. By applying such a method to the parathyroid glands from human new-born, typical cultures could indeed be obtained (Kooreman and Gaillard, 1950; Gaillard, 1953).

In practice, for the parathyroid gland from human new-born, a completely homologous medium had to be used, and the following medium was found empirically to be adequate: human blood plasma, 1; human cord serum, 2; foetal human brain press juice, 2; balanced saline solution, according to G.O. Gey. 15.

As a rule, embryonic watch glasses were used and 5–8 tiny fragments (±0·5 mm.³) of parathyroid tissue were placed on top of the medium immediately after coagulation had occurred.

Placed in an incubator at 37°C., within twenty-four hours the coagulum just around the explants became liquefied and in this way a little pool of fluid medium was created. Soon

after the liquefaction had occurred, epithelial cells migrated along the borderlines of the explants and in this way encapsulation by parenchymal cells occurred (see Fig. 1).

Every third day the explants were sucked up in a pipette washed in a balanced saline mixture and placed on top of a fresh medium.

During the cultivation procedure most of the explants gradually increased in volume, due to the apposition of newly formed epithelial cell layers and enlargement of the cell nests in the interior of the fragments This enlargement seemed to be due for the greater part to an increase in the cell volume rather than to mitotic cell division (see Fig. 2). Moreover, only clear cells appeared to be present.

As long as the "typical parathyroid structure remained intact the medium always became liquefied, this occurred for sixty days at the longest.

Throughout the procedure great care must be taken to avoid injury to the enclosing epithelial membrane, for the latter is essential for a successful organized growth.

The above results led to the use of human parathyroid explants in replacement therapy in patients suffering from post-operative tetany. Stimulated by the work of Stone, Owings and Gey (1938), the perivascular lymph spaces around the axillary artery or vein were used as the site of transplantation.

In order to transplant the parathyroid tissue the technique just described was modified according to principles described by Erdmann (1927) and Gassul (1923), in an attempt to "adapt" the explants to the humoral milieu of the future host Accordingly, the medium was changed gradually and in such a way that after one week of cultivation in the medium described above, the blood plasma and the placental vein serum were replaced stepwise by the plasma and serum of the acceptor After this had been done the brain press juice was completely omitted. After fourteen to twenty-one days the explants were considered to be ready for transplantation

The patients were investigated clinically and chemically



FIG 1 Explants of a fragment of the parathyroid gland from a newborn human, cultivated for ten days In the centre some cell nests are to be seen Note the parenchymal encapsulation of the fragment

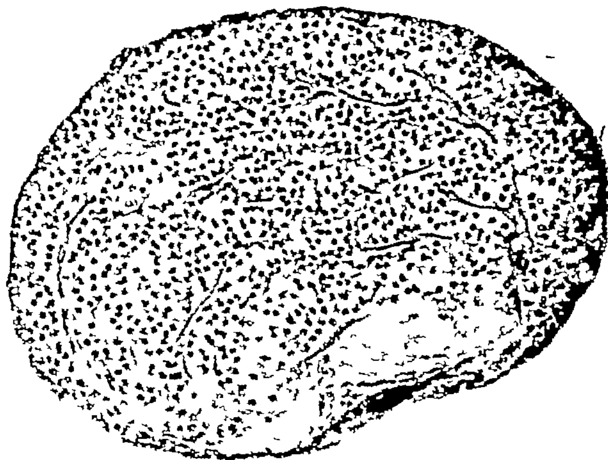


FIG 2 26 day-old parathyroid gland from a newborn human. As compared with Fig 1 a considerable increase of "clear" cells has occurred Thin strands of connective tissue separate the nests of "clear" cells

Number	Patient	Year(s) of strumectomy	Year(s) of transplantation	Age at the time of transplantation	Result at 1 January 1952	Sex
1	v D	1942	1948, 1949	17	negative	F
2	v L	1940	1944	18	+since 1944 (8 years)	F
3	v d H	1942	1943	19	+since 1943 (9 years)	M
4	G K	1948	1951	19	not judged	F
5	Be	1941	1942, 1943, 1944	18, 19, 20	+since 1944 (8 years)	F
6	Hey	1938	1948	23	negative	F
7	v Rij	1947	1949	23	negative	F
8	W	1940	1948, 1950	25	negative	F
9	B S	1941	1943	25	+since 1943 (9 years)	F
10	Mi	1939	1942	26	died	F
11	St	1942	1947	27	negative	F
12	v d R R	1938	1941	27	+since 1941 (11 years)	F
13	S de K	1939	1949	27	+since 1949 (3 years)	F
14	H D	1944	1947, 1948	27, 28	partial result (4 years)	F
15	v O B	1939	1947	28	negative	F
16	M R	1936	1947	29	negative	F
17	de Vi	1937	1941, 1944	27, 30	+since 1944 (8 years)	M
18	Ku	1940	1943, 1944	29, 30	negative	F
19	T F	1936	1946	31	negative	F
20	Ko	1939	1946	32	1946–1950 positive (4 years) since 1950 partial result	F
21	B v d L	1942	1943	35	unknown	F
22	Hes	1944	1947	36	negative	F
23	S v d L	1942	1943	37	negative	F
24	Val	1940	1947	38	negative	F
25	Kl	1939, 1941	1943, 1944	41, 42	negative	F
26	v G	1945	1946	45	negative	F
27	S P	1942	1943	45	negative	F
28	Me	1927	1940, 1940, 1941, 1942, 1947	41, 41, 42, 43, 48	negative	F
29	M v d K	1936	1940, 1943	45, 48	negative	F
30	V S	1945	1946, 1948	50, 52	negative	F

before and after the transplantation Table I summarizes the results obtained up to 1st January, 1952.

It is evident that there are a number of long-term positive results; transplantation of cultivated parathyroid gland fragments from the new-born child into patients suffering from a post-operative tetany *can* be successful. It should be noted, however, that all the positive cases are concentrated in the upper part of the table, which means that only the younger patients appear to have had some chance of recovery. No patient older than thirty-two years of age shows a positive

Table II

EFFECT OF AGE ON SUCCESS OF PARATHYROID TRANSPLANTATION (SAME CASES AS TABLE I)

Age in years	*Total number*	*Cured*	*Partially cured*	*Not cured*	*Peculiarities*
16–26	9	4	0	4	One transplantation was done only some months ago
26–36	12	3	2	5	One patient died, one patient disappeared
36–46	6	0	0	6	None
46–56	3	0	0	3	None
Totals	30	7	2	18	3

reaction and consequently the interaction graft-host seems to change during postnatal development

This point can perhaps be demonstrated more clearly by dividing the cases into arbitrarily chosen ten-year groups, as has been done in Table II

It can be seen from this table that changes seem to occur in the graft-host interaction, suggesting that the probability of a successful graft *gradually* decreases during postnatal life. That changes occur can also be demonstrated by the fact that repeated transplantation in younger patients sometimes led to positive results, while in older patients this procedure has never resulted in a positive reaction. Unfortunately the results obtained to date do not indicate what kind of changes

are responsible for the different results in older and younger patients, and further research on this particular point seems necessary It is interesting to know that analogous results have been reported in animals by Loeb and King (1935), and so the occurrence of this change may indicate a general principle.

Finally, we wish to stress that no histological proof of viability is available, but on the other hand the positive results so far obtained do not seem to be easily explained unless the parathyroid tissue is alive.

Considering the work of Sanford and Algire (1952), one might even doubt whether the tiny fragments introduced in the meshes of the perivascular lymph spaces are vascularized at all, as these authors claim that in their experiments survival of homotransplants appeared to be possible only so long as no vascular supply developed.

As to the rôle of the culturing procedure, there is no absolute proof that it is necessary. We can only say that some positive results have been obtained and that other authors have not described comparable long-term results with non-cultured material The culturing procedure certainly seems to be of importance for the selection of the most regenerative fragments which, after having been encapsulated, become structurally adapted to feeding by diffusion At any rate, a balanced condition of the tissue has been obtained at the time of transplantation.

Finally, the tissues of the newborn must be considered to possess a low degree of antigenicity and, according to some recent experiments by Langman (1950), some "adaptation" might really have occurred.

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DISCUSSION

MEDAWAR: I should like to ask the opinion of Prof Gaillard and the other tissue culture experts here on one possible explanation of why cultivating grafts of this kind *in vitro* may make them work particularly well as homografts It is well known that all epithelial cells *in vitro* have a polarity, and in general we can say that they get their food and their oxygen, or most of it, from the stromal side, while their secretory products, if any, pass out at the opposite pole. But although most of the food and oxygen, etc presumably gets in through the inner pole of the cell, there is a certain amount of evidence that a good many epithelia can get nourishment from the outside as well I'm thinking in particular of the corneal epithelium, which appears to get some of its oxygen from the outside (there has been some very interesting American work on the anoxia caused in the corneal epithelium by using contact lenses). Now if you cultivate small epithelial organ fragments, little cysts tend to be formed In the case of skin or cornea these cysts may be of two sorts either with the epithelium on the outside and the residue of the stroma on the inside, or cysts in which the entire polarity is reversed, so that you get the epithelium on the inside and the residue of the stroma on the outside If by preparatory treatment in tissue culture you tend to get a closed cyst of the first type, with the normal outer surface of the epithelium presenting outwards, it may perhaps be particularly well suited for survival as a homograft It may be nourished—perhaps in a not very effective way—by diffusion from the outside, and it does seem reasonably likely that a cyst of this sort, with the vulnerable inner poles of the cells shut off from the environment, would be particularly resistant to the action of antibodies It's just a possibility that by tissue culture you are "training" the cells, but in a histological rather than a biochemical sense

GAILLARD The only thing I can say is that during the *in vitro* cultivation the parathyroid explants sometimes develop a capsule of connective tissue, and in those we were never successful But there were only a few cases

LONGMIRE With regard to the length of time that these patients showed evidence of calcium deficiency before being grafted I think all clinicians are aware that it is not uncommon for certain patients following thyroidectomy to undergo a transient period of hypopara-

thyroidism which may be severe and may last for several months It is assumed that this is probably due to interference with the blood supply of the remaining parathyroid tissue The blood supply is re-established at some later time, the tissue develops and is again able to take care of the bodily needs So it is important in a series of this kind to indicate the length of time the patient had evidence of hypoparathyroidism

GAILLARD We waited until at least one year after strumectomy In most of the cases there were about two to three years between the strumectomy and the grafting

POMERAT It is important I think to be clear about the nature of the experiments done by Gey and Stone They were using a disorganized sheet, whereas you are using a whole organ maintaining its organotypic picture The second point is, what about the adaptation of that organ's medium to the prospective host?

GAILLARD: During the last ten days before grafting we cultured the fragments in a medium which was completely derived from the future host Now I don't know if this means anything, but Dr. Langman in our Department, who has recently been working in the Anatomy Department in Amsterdam, has cultured ovarian tissue from rabbits in the plasma of cats With the help of a microflocculation reaction, he was able to demonstrate that after a fortnight of cultivation, cat antigens could be demonstrated in those rabbit fragments I'm not quite sure if one may speak here of a real adaptation All I can say is that the antigens couldn't be washed out by five rinsings with a balanced saline solution

KROHN Have you ever attempted to find out if there is any parathyroid hormone in the fluid of your culture media?

GAILLARD No We did something of this kind with insulinoma cultures After fourteen to twenty days of cultivation we found by injection into rats and studying the hypoglycæmic effects that there was a large amount of hormone in the medium, but I can't say whether it was newly produced or just liberated from the fragments

EARLE In working with tumour tissue I know it is customary to implant into the subcutaneous tissue It has been our experience, however, that such implantations are far inferior to intramuscular implantations I wonder whether these implantations of parathyroid would not be superior and longer-lasting if they were implanted into muscular tissue

GAILLARD We have tried intramuscular implantation in dogs, but we have not had as good success as in the perivascular lymph spaces From a recent series of experiments with grafts of the ovaries from newborn mice in adult males it became clear that the site of the transplantation is of great importance for the result So far the results of grafting in the perivascular lymph spaces and under the capsule of the kidney appear to give far better results than with subcutaneous, intratesticular and intramuscular grafts Originally we thought that these differences might perhaps be explained by differences in the vascularization, because it has been demonstrated by Sanford and Algire that small fragments of thyroid gland can remain alive and function

for a year or so, provided that no vascularization occurs. All our successful grafts, however, appeared to be intensively vascularized, more or less independently of the sites of transplantation used so far

EARLE What is the approximate size of the fragments?

GAILLARD About 1 mm in diameter, sometimes a little bigger, sometimes a little smaller These parathyroid fragments are hard to prepare; one has to take all the little organs in the environment of the thyroid gland, culture them all, and after two days it turns out which is which.

EARLE I find the size of these clumps of great interest. If you cut your clump too small, the injury to the clump by the surrounding solution—assuming that you're using saline—is going to be rather severe If you cut your clump too large you're going to get central necrosis For injection I expect these clumps are about the ideal size to protect the cells from the saline On the other hand if they are 1 mm in size, you're almost surely going to get a good deal of central necrosis unless there is fairly rapid vascularization, that is, assuming the tissue continues to have a reasonably high Qo_2

With respect to what Prof Medawar was saying about the polarity of the cells, in our skin epithelium cultures, the epithelium exists first as a massive sheet in the culture Gradually the cells migrate out from it in the form of a very thin sheet, then the sheet breaks up and finally you have cells all over the surface area, the cells in their morphology being practically indistinguishable from what we call fibroblasts. I doubt very seriously whether those cells can be thought of as oriented with respect to their secretion, because I think that all dimensions of the cells are within the range of diffusion from one cell to another cell

MEDAWAR The sort of culture I had in mind was fluid flotation culture—hardly tissue culture at all in Dr Earle's sense. Fluid flotation cultures of tiny little fragments of skin do indeed form cysts of the one sort or the other The conditions Dr Earle is describing are expressly designed to favour disseminated growth

EARLE It is of interest that in our fluid suspension cultures that I described yesterday, by reducing the rate of rotation of the tube we were able deliberately to produce spherical masses ranging, as desired, from 0 1 mm. up to 1 mm in diameter

ROGERS Dr Earle, must we assume that in order for Dr Gaillard's parathyroid tissue to be effective it must be vascularized, or may we assume that in the perivascular lymph spaces this tissue is existing in what we might call a lymphatic or a plasmotic circulation, and needs no inherent vascularization?

EARLE If the Qo_2 of this parathyroid tissue in the lymph spaces is reasonably high, I would find it very hard to believe that a densely cellular spherical mass, about 1 mm in diameter, can exist for any length of time without very effective vascularization In Warburg's original research, the effective thickness of tissue having a reasonably high Qo_2, assuming diffusion from both sides, is of the order of 0 2 mm Our observations agree with Warburg's in that I do not believe a sphere of roughly 1 mm in size with a reasonably high oxygen consumption could persist without revascularization It would show central necrosis

or else a diffused necrosis which would result in an effective reduction of the Qo_2 of the mass as a whole

ZUCKERMAN Does the central mass regenerate if central necrosis has occurred in the fragments which Prof Gaillard is implanting into the perivascular lymph spaces?

EARLE I think you might have effective regeneration within any area that could be adequately oxygenated either due to the limited size of the mass or else to vascularization Without vascularization and assuming a reasonably high Qo_2 of the tissue I would not look for living cells to continue viable more than several tenths of a mm removed from the surface If the tissue mass is vascularized, then I think you can carry it up to any size that you want.

GAILLARD I might say that sometimes in culture conditions we find in the interior of the explants a collagenous mass, with a cortex of active parathyroid tissue around it As a rule such pictures do not indicate that a central necrosis originally occurred On the other hand there are quite often much bigger explants, consisting of beautiful parathyroid epithelium, which obviously can remain alive for many weeks without any vascularization at all

THE BEHAVIOUR OF EMBRYONIC ENDOCRINE HOMOGRAFTS:

An experimental study of adrenal grafts, with notes on an ovarian graft in a young woman.

P F. JONES

The transplantation of pieces of bone, a length of artery, or the cornea, from one adult to another is now an established surgical procedure having both clinical and experimental sanction. There may be doubt about the eventual fate of such grafts but they will function over long periods There is equally strong evidence that the transference of skin or a kidney from one person to another can only be of temporary benefit Such basic knowledge of the behaviour of homografts is necessary in order to know which tissues are worth preserving for transplantation, and in the case of endocrine tissues this knowledge is incomplete. It is my purpose to review this subject briefly and record some fresh experiments.

The truth of "Halsted's principle" has been repeatedly confirmed and all grafts reviewed and recorded here have been made in subjects with an endocrine deficiency

Homografts of Adult Tissues

The common view is that endocrine tissue from a healthy adult transplanted into an adult with an endocrine deficiency state cannot be expected to survive, and most of the evidence supports this opinion Ingle, Higgins and Nilson (1938) removed the adrenals of 26 adult rats and inserted into them adrenal grafts from unrelated adults no successful grafts resulted. Higgins and Ingle (1938) performed a similar experiment on 15 adult rats, taking grafts from unrelated rats two months old, and there was only one survivor with a

functioning graft Woodruff and Woodruff (1950) implanted homografts of adult thyroid tissue into 37 thyroidectomized unrelated guinea-pigs when the graft site was explored twenty-eight to thirty-five days later healthy thyroid tissue was found in only four animals. Loeb (1930) states that, in his experience, it is rare for homografts of thyroid tissue to survive. A small experiment of my own, in which the adrenals of ten adult rats from one laboratory were exchanged with the adrenals of ten adult rats from another laboratory, did not result in any successful grafts The report of Stone, Owings and Gey (1934) is exceptional. 11 dogs received homografts of thyroid which had been cultured in the recipient's serum and five survived with functioning grafts There is no note of the relationship of host to donor. The experiments of Smith and Parkes (1951) suggest that homotransplants of the ovary may survive more easily than those of other endocrines, but Blair Bell (1925), who had a considerable experience of ovarian grafting, considered they were very rarely effectual The results of other clinical workers are not encouraging. Out of a number of reports of adrenal grafts only Pybus (1924), Goldzieher and Barishaw (1937) and Broster and Gardiner-Hill (1946) seem to have achieved any real success In two of these cases the graft came from patients with the adreno-genital syndrome

Homografts of Embryonic Tissues

There has been much debate whether the behaviour of embryonic endocrine homografts differs from that of adult glands Higgins and Ingle (1937) record the only useful experimental series They adrenalectomized 21 adult rats and implanted the adrenals of unrelated new-born rats nine survived for four months, and in each a nodule of healthy adrenal cortical tissue was found at the site of the graft. A similar experiment in which 40 adult rats were grafted with the adrenals of new-born rats of "distant relationship" resulted in 24 functioning grafts Using baby rats of increasing age as donors lowered the number of successful grafts.

Hurst, Tanner and Osman (1922) and Bailey and Keele (1939) record one case each of Addison's disease successfully treated with a neonatal graft, although in judging these results the experience of Woodruff (1952) must be remembered. He grafted three patients with Addison's disease with fœtal adrenal tissue and great clinical improvement followed, but he failed to find any trace of the grafts when the patients were explored six to nine months later. Kooremann and Gaillard (1950) record a series of 17 patients with hypoparathyroidism treated by grafts of cultivated neonatal parathyroid tissue They had seven successes, all in young patients.

Present Experiment

The results quoted suggest that embryonic endocrine homografts take more readily than grafts of adult glands and the experiment recorded here was designed to provide further information First, in the experiments reviewed, absence of relationship between donor and host was not always certain; in this experiment the grafts were made from donors who could not be related to the hosts Second, there are no records of the survival of experimental homografts beyond four months. In this series the grafts of the majority of survivors have been inspected but not removed and their behaviour over longer periods has been observed. Lastly, it is not yet clear whether one site of implantation for free grafts is better than another. This series compares three possible sites of graft implantation.

Methods

The hosts were fully grown albino rats All were over the age of nine months and most were more than fifteen months old The donors were rats within forty-eight hours of birth: they were bred in a different laboratory from that supplying the strain of rats which were the hosts.

The donors were sexed, killed by ether anæsthesia and the adrenals removed and placed in normal saline at room temperature. These glands were implanted within two hours



Fig 1 A neonatal adrenal graft on the surface of the erector spinæ of the adult rat, seven months after implantation The graft consisted of two split adrenals $\times 2\frac{2}{3}$



Fig 2 Neonatal adrenal graft alongside the femoral vessels, four months after implantation The femoral vessels run nearly vertically down the centre of the photograph, the superficial epigastric vessels rise just to the left of the graft The graft

Table I

SUMMARY OF RESULTS, SHOWING NUMBER OF ADRENALS GRAFTED AND THE SITES USED FOR IMPLANTATION

	Animals grafted	*Survivors with grafts established*
1 or 2 adrenals		
surface of erector spinæ	37	7
3 to 6 adrenals		
surface of erector spinæ	16	5
femoral sheath	15	4
wound in erector spinæ	18	6

is shown in Table II. Those animals which have survived the longest are in excellent health and they have recently been re-examined. The grafts look healthy and vascular and, particularly in view of the fact that death has always followed excision of such grafts, I believe the grafts are maintaining the life of these animals.

Care was taken to perform radical adrenalectomies and no examples of survival due to the presence of accessory adrenal tissue have been seen. Sixty-four animals have died after the insertion of grafts—usually one or two weeks after stopping saline drinks. Post-mortem examination has shown either

Table II

SUMMARY OF SURVIVORS

Animals surviving	*Length of time*	*Result*
1	11 months	Alive
5	9 months	Alive
1	8 months	Dead (graft healthy)
1	8 months	Dead (no P.M.)
2	5½ months	Dead (grafts degenerate)
2	5 months	Alive
1	4 months	Dead (graft healthy)
1	3 months	Dead (graft healthy)
1	3 months	Dead (graft degenerate)
2	2½ months	Grafts healthy when excised at these times
2	2 months	
1	1 month	

complete absorption of the grafts, a nubbin of granulation tissue which on section contains no adrenal cells, or the grafts lying dead but macroscopically unchanged This last finding was noted in 24 animals and is rather striking when found three to four weeks after the implantation of the grafts. It seems that the grafts have aroused no reaction in the surrounding tissues, and the failure of the grafts is due to this rather than to the production of a local phagocytic reaction If embryonic tissues produce so little reaction in the tissues, the development of an adventitious blood supply will be delayed and this was the reason behind the use of a muscle wound as a grafting site So far there is no evidence that it is a better site than the others, but the matter of the best site for a free organ graft needs further study.

These experiments showed that from one-quarter to one-third of embryonic endocrine grafts in rats could survive and function for periods up to eleven months, that their growth was controlled and they remained encapsulated. It therefore seemed right to extend this method of treatment to patients.

Clinical Applications

The supply of donors of material for human homografts is generally limited and fœtal material provides no exception to this experience. With modern obstetric care comparatively few terminations of pregnancy after the eighteenth week, or fresh stillbirths, occur, and until reliable methods of preservation of endocrine tissues are developed it will be necessary to bring the donor and the host together very soon after delivery. This may require long waits culminating in hasty arrangements and nocturnal operations, for it seems desirable to transplant fœtal endocrine material within two to three hours of death. Owing in part to these administrative difficulties I can only report on the treatment of one patient by the grafting of human fœtal material

A married woman of thirty-four years had a bilateral oophorectomy and hysterectomy performed four years before

she presented with severe menopausal symptoms: the most serious of these were frequent hot flushes with excessive sweating of the palms, and attacks of severe vaginal pain due to an advanced senile vaginitis. Hormone replacement therapy had failed to relieve these symptoms.

Apart from the listless and dowdy appearance of the patient, the objective changes were a loss of fat from the labia majora, kraurosis vulvæ and a senile vaginitis. Neither squamous cells nor glycogen could be demonstrated in vaginal smears

A full explanation of the proposed graft was made to the patient and she willingly agreed to its being done. As she lived outside London she remained in hospital and finally had to wait six weeks before suitable material was obtained from an embryo at the twenty-sixth week of fœtal life, delivered by abdominal hysterotomy. Both ovaries were removed, dropped into normal saline and taken to the theatre where the patient was ready A midline subumbilical incision was made, both rectus sheaths opened, the ovaries chopped into pieces 2 to 3 mm. in diameter and spread over the surface of both rectus muscles. This operation was completed within one hour of the delivery of the child. The wound healed normally. On the twelfth post-operative day the patient complained of vaginal irritation and a vaginal smear showed some glycogen to be present and a few squamous epithelial cells were seen.

Four weeks later the graft site felt normal, the vagina looked healthy, the signs of kraurosis had gone and a stained vaginal smear had a good glycogen content. There was a striking improvement in the appearance of the patient, which was not only due to her purchase of a new costume and hat. When last seen, five months after the graft, improvement in this patient was maintained There was complete relief from the symptoms and signs of senile vaginitis and kraurosis, and the vaginal smear showed some squamous epithelial cells and contained glycogen

This case illustrates the difficulties of obtaining fœtal and

neonatal material and gives point to the need for a reliable method of preservation of endocrine tissues. It shows that this type of graft can be of practical value, for in this patient it provided a useful answer to what had become a difficult therapeutic problem. It is impossible to say whether human homografts will behave in a similar way to those in animals. At present I would not claim that there is more than a small chance that a given embryonic endocrine graft will survive and function, but at least the procedure is harmless and it may be most valuable I believe that the results of the work on the behaviour of these grafts fully justify an extension of this form of treatment to suitable patients.

Summary

Eighty-six adrenalectomized adult rats received adrenal grafts from unrelated new-born rats. Twenty-two rats survived on a normal diet and functioning grafts were demonstrated Details of the sites available for grafting and of the length of survival after grafting are given.

These investigations show these grafts to be harmless and the use of this method is justified in suitable patients. A successful fœtal ovarian graft in a young oophorectomized woman is described.

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DISCUSSION

ZUCKERMAN Was the section of the adrenal cortex that you showed typical of all regions of the grafts? I ask because the cells are not arranged in the normal fasciculate way, and I am wondering what significance should be attached to this

JONES This illustration was a section of a graft one month after implantation. Sections of older grafts, say at ten weeks (Fig 4), do show a clear zona glomerulosa and fasciculata.

ZUCKERMAN I take it that you insert these fœtal adrenals complete with capsule.

JONES: Yes, I made a point of leaving the capsule on.

ZUCKERMAN. Would you get much the same result if you cleaned out the contents and just inserted the capsule?

JONES The only paper I know on this subject clearly suggests that you would

ZUCKERMAN: That is why I asked about the organization of the fasciculata, because it seems to me that a normal arrangement of the fasciculata would imply that blood vessels were growing in from the periphery.

PARKES In practice, where we have used adult adrenals, we have cut the single gland into two, enucleated the two halves and implanted the capsule. That works beautifully with autografts, we have not much experience of homografts

DEANESLY Our grafts mostly show organization of the cortical cells when they've grown—certainly more organization than is shown in Mr. Jones's figure

PARKES But we have never used grafts as young as his.

DEMPSTER. Do you know the lipoid contents of your grafts?

JONES: No

DEANESLY. Did you make a regular search for accessory adrenals?

JONES At each post-mortem I have looked for them, but have never found any. I have always made a point of doing a very radical adrenalec-

tomy I am morally certain that none of the surviving rats had any, because a healthy graft was found in each one

PARKES You have been rather lucky We are plagued with about 10 per cent accessory adrenals Our procedure for testing frozen adrenal tissue is complicated by the fact, it picks out beautifully the animals with accessory adrenals They are the ones that survive

DEMPSTER I was interested to see that there was no local reaction around your grafts Was that typical of all of them?

JONES Yes, I have never seen any other appearance

DEMPSTER Did you observe degeneration?

JONES Once the graft has degenerated one may see obvious foreign body reaction granulation tissue and a certain number of foreign body giant cells

DEMPSTER And how about adult adrenals?

JONES I have never found any trace of the grafts when animals given adult adrenal grafts died and come to post-mortem I have made no histological observations in these animals

DEMPSTER. I think you've posed a very interesting concept of the problem of the "take", which I think is bound up very intimately with the whole of Halsted's law You were implying, I think, that if there was no local reaction there was no revascularization Am I right in saying that?

JONES I believe that to be a fundamental point

DEMPSTER I agree with you so far I would say that the revascularization of an implant is a purely fortuitous phenomenon. If you inject the graft either with a needle through the skin or host tissue—that, in the rat, is enough to stimulate the small blood vessels it is certainly so in the case of thyroid grafts I think the whole problem of Halsted's law turns on that very point of revascularization That is what we mean by a "take". But I don't know what Halsted's law means in terms of grafting an endocrine organ with a blood supply, where there's no question of actual "taking"

MCLAREN Do you get a strong local reaction when grafting these embryonic glands into non-adrenalectomized rats?

JONES I have never tried it

KROHN In the case of the ovariectomized woman that you described, you said, I think, that she had had severe menopausal symptoms, and that hormone treatment had failed to influence them

JONES I believe the heat flushes were slightly reduced by this treatment, but it had very little effect on her vaginitis

KROHN What then do you think the ovarian graft produced which was responsible for these changes? Is it something entirely different from the hormones that we normally think about?

JONES I'm certainly not qualified to say

DEMPSTER Was the patient made aware of the nature of the operation and its effects?

JONES I explained to her what would be done, but I said nothing about the effects, as I had no idea what they would be I said there was a rather small chance it might benefit her

DEMPSTER: Because there can be very striking psychological effects, we know that the abdomen can swell in certain psychological states in females who are not pregnant. After all, Voronoff made a living by grafting simian testis!

JONES: This woman was certainly very depressed and she was greatly encouraged by having this graft made. But at the same time you can hardly explain her changed vaginal smear on a psychological basis.

ZUCKERMAN: There is a possibility that in the post-operative phase we are dealing with an increased production of œstrogen by the adrenal cortex. When the ovaries are removed either from a rat or a monkey (and I believe similar observations have been made in human beings), cyclical vaginal changes do continue. It is conceivable that an ovarian graft might react on the pituitary in such a way that the adrenal cortex would produce more œstrogen than it may normally do.

JONES: I'm hoping that estimating the gonadotrophins may help. I should imagine that if there is a functioning ovary it may depress their secretion.

ZUCKERMAN: It might, but one must remember that ACTH, for example, is not necessarily specific in its effect on its target organ. It is possible that gonadotrophin estimations will not provide the complete answer.

PRESERVATION OF BLOOD

H CHAPLIN, Jr and P L MOLLISON

MANY of the constituents of plasma can be satisfactorily preserved for long periods, for example, gamma-globulin can be kept for years after freeze-drying and confer temporary passive immunity on a subject into whom it is injected Even quite labile elements, like anti-hæmophilic globulin, can be stored for long periods provided that the plasma is frozen within twenty-four hours of withdrawal from the donor

Methods for the preservation of the formed elements of blood are less advanced When blood is mixed with citrate in ordinary glass containers and stored at 4°C the red cells remain viable for a time but the white cells and platelets do not Methods of storing viable white cells and platelets are being developed (Tullis, 1953*a*, 1953*b*). However, white cells survive for only very short periods after transfusion (Lawrence, Ervin and Wetrich, 1945, Weisberger, Heinle, Storaasli and Hannah, 1950) and the value of platelet transfusions is limited (Sprague, Harrington, Lange and Shapleigh, 1952) By contrast, fresh red cells, when transfused from one subject to another, usually survive as long as do the recipient's own red cells Red cell transfusions thus have a wide scope and the preservation of red cells for long periods is a desirable objective. This paper describes some progress that has been made in this field

The first step in red cell preservation was made by Rous and Turner when they found in 1916 that storage of blood with an excess of citrate glucose solution led to a maintenance of viability of the red cells for at least two weeks, they were able to show that after bleeding rabbits, and substituting stored red cells, hæmoglobin concentration was well maintained Slight further progress was made during the second

world war when it was shown that adjustment of the pH of blood-citrate-glucose mixtures to about 7·2 appreciably prolonged the storage life of the red cells (Loutit, Mollison and Young, 1943) It should be emphasized that despite extensive studies it has never become possible to predict from tests *in vitro* how the red cells will survive *in vivo*. For example, red cells stored in a citrate-sucrose mixture undergo very little lysis during a three week period of storage at 4°C., as Rous and Turner noted, but when the cells are transfused they are rapidly swept from the circulation (Mollison and Young, 1942).

The chief changes which occur in red cells stored in sodium citrate at 4°C. are firstly, loss of potassium and gain of sodium The gain in sodium exceeds the loss in potassium, leading to a slight increase in total base, and this is thought to be the cause of the swelling; however, it is not the sole cause of the increase in osmotic fragility, for the "critical bursting volume" diminishes progressively during storage (Rapoport, 1947). Secondly, there are changes indicating that metabolism continues at 4°C., for example, a diminution in the cell content of dextrose and of hydrolysable phosphate (Maizels, 1941; Rapoport, 1947).

All these changes are reversible, for red cells which show them may survive normally after transfusion. Nevertheless, it is thought that the changes are symptomatic of progressive deterioration for there is a rough parallelism between the extent of the changes *in vitro* and the impairment of survival *in vivo* (Maizels, 1943, Rapoport, 1947).

The lysis of red cells stored at 4°C. is not due to swelling and bursting but is probably due to changes in the cell membrane (Mollison and Young, 1942; Maizels, 1943). It has been generally assumed that these changes indicate the activity of metabolic processes without repair and that better red cell storage can be achieved only by interfering with metabolism, either by the addition of substances which block enzymes or by lowering temperatures to a point where metabolism is greatly slowed.

It may be interesting to discuss one example of an attempt to improve preservation by adding an enzyme inhibitor, because it illustrates the essential rôle which transfusion experiments play in assessing the state of preservation. Halpern, Dreyfus and Bourdon (1950) discovered that phenergan and a related phenothiazine derivative, "RP 3300," would delay hæmolysis when added to blood stored at 4°C.; moreover, cell swelling and potassium loss were delayed. It was concluded that these agents were tissue preservatives and that this was probably due to their power of inhibiting enzymes, particularly cathepsins and tryptic enzymes.

Experiments made at the Blood Transfusion Research Unit showed that although the addition of RP 3300 delayed lysis and delayed swelling, survival after transfusion was not improved (Chaplin, Crawford, Cutbush and Mollison, 1952) The method of studying survival was a modification of the method originally described by Ashby (1919). Recipients of group A, type M received group O, type M red cells (control) and group A, type N cells (test). After transfusion, red cell counts made in the presence of anti-M serum enabled the A N cells to be counted and with anti-A serum the O M cells could be counted Each recipient received test red cells—stored in the presence of RP 3300—and control cells, stored for the same length of time without RP 3300 These experiments showed clearly that red cells stored with this compound did not survive any better after transfusion and that the changes observed *in vitro* had been wrongly interpreted.

The possibility of storing red cells at low temperatures was much discussed after the war but little was done until Dr. Parkes's collaborators demonstrated that relatively large amounts of red cells could be frozen and thawed without lysis if glycerol were present (Smith, 1950). Sloviter (1951*b*) obtained evidence that red cells recovered after freezing at —79°C. and thawing were viable in rabbits, and transfusion experiments in man confirmed this (Mollison and Sloviter, 1951). In these experiments blood was mixed with glycerol-saline to give a final concentration of glycerol of approxi-

mately 15 per cent w/v. It was found that after storage at −79°C. for six months as many as 50 per cent of the red cells might be lysed but that the recovered red cells survived normally (Mollison, Sloviter and Chaplin, 1952) Similar experiments were also done at −20°C. and showed that although lysis progressed rapidly at that temperature, so that in some experiments as few as 20 per cent of the cells were recovered after three months, nevertheless the recovered cells survived normally. From these experiments it was concluded that metabolic changes were arrested, even at −20°C., and that if only some method of reducing progressive hæmolysis could be discovered, storage of blood by this method would become practicable.

In the course of experiments on the effect of glycerol concentration on the optimal rate of freezing it was discovered that for blood frozen in bulk to −79°C. there was far less lysis when a glycerol concentration of 30 per cent rather than 15 per cent was used. Moreover, at this glycerol concentration the rate of freezing was no longer critical, and bottles of blood could be frozen by simply plunging them into a solid-CO_2 alcohol mixture (Chaplin and Mollison, 1953) In other experiments it was found that red cells mixed with a sodium citrate-glycerol solution underwent far less lysis on being stored at −20°C. than did cells mixed with saline-glycerol solution Packed red cells mixed with an equal volume of 3 per cent trisodium citrate and sufficient glycerol to give a final concentration of 30 per cent w/v after mixing with the red cells underwent only about 2 per cent lysis in three months (Chaplin and Mollison, 1953).

At this stage it appeared that really useful conditions had been found However when red cells stored at −20°C. in such mixtures were transfused, survival was found to be poor. For example, after three months' storage, the percentage survival twenty-four hours after transfusion was about 50 per cent. Red cells stored in the same mixture at −79°C survived much better after transfusion but apparently not as well as red cells stored at this temperature in a glycerol-

saline solution (Chaplin, Crawford, Cutbush and Mollison, 1952)

These findings led to making tests *in vitro* on red cells stored in mixtures containing 3 per cent trisodium citrate and 30 per cent glycerol It was found that after storage for three months at −20°C there was an astonishing loss of potassium without an equivalent gain in sodium, so that the red cells had only about 70 m Eq of base per litre instead of about 115 m Eq There was an associated reduction of cell volume As expected, such cells showed a considerable decrease in osmotic fragility

It does not seem likely that mere depletion of potassium impairs survival *in vivo* for it is known that cells poor in potassium and rich in sodium can recover almost normal values within twenty-four hours of being transfused (Maizels, 1943) The loss of total base and the excessive shrinkage may indicate damage to the red cell membrane, but there is no direct evidence on this subject In this connection it is of some interest that red cells stored in citrate-glycerol mixtures for three months at −20°C are well agglutinated by blood group antibodies, a fact which makes this method of storage very convenient for blood group laboratories

A point of great interest is to determine to what extent metabolism continues at −20°C It is a fact that red cells stored at −2°C survive after transfusion no better than red cells stored in the same mixture at +4°C. (unpublished observations), if the deterioration occurring at +4°C is due to metabolic activity, then lowering the temperature by 6°C is insufficient to reduce this activity to a material extent and it is doubtful whether reduction by another 18°C will stop all activity This implies that much lower temperatures may be needed for satisfactory long storage.

On the other hand the fact that even at −79°C there appears to be some deterioration in citrate-glycerol mixtures, and the fact that −2°C does not seem to be better than +4°C when acid-citrate-dextrose mixtures are used, suggests that the damage is at least partly physical and that it may be

possible to preserve red cells for very long periods when the nature of this deterioration is better understood

It should be emphasized that the studies referred to so far have been made on cells from which the glycerol has been removed after the period of storage. At first dialysis was used (Sloviter, 1951*a*); this was effective but cumbersome and time-consuming. Then the method of Lovelock (1952), in which the glycerol is "sucked out" of the cells by the addition of hypertonic citrate, was tried, this was very suitable when concentrations of glycerol of the order of 15 per cent were being dealt with but was not satisfactory when the concentration was as high as 30 per cent The method we are using at present is simply to wash the red cells in falling concentrations of glycerol; for example, the cells are washed in solutions containing 16 per cent, 8 per cent and 4 per cent glycerol in 3 per cent trisodium citrate and are then washed three times in saline It was suspected that so much washing might impair the viability even of fresh red cells. However, transfusion experiments have shown that seven washes of red cells with twice their volume of normal saline does not impair their subsequent survival *in vivo* (Chaplin, Crawford, Cutbush and Mollison, 1954)

The present method of washing is very cumbersome, seven separate centrifugations being necessary. However, considerable progress has been made in the development of continuous washing devices (Chaplin and Veall, 1953). The type we are using at present is essentially that described by Dr. Ivan Brown of Duke University, U S.A , in which the red cells are washed in their original containers. Thus when more satisfactory conditions for the long storage of red cells at low temperatures are found, an apparatus for preparing them for transfusion with very little trouble should be ready.

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DISCUSSION

MEDAWAR Could I ask a question about the assay method used for finding out how long these red cells survive? You are, of course, starting with a population of cells of mixed ages, and I would think that some of your results would suggest, purely from the mathematical point of view, that in the course of storage you're getting a differential mortality of cells of different initial ages Are the older cells more fragile? And have you tried, for example, taking a pint of blood and incompletely hæmolysing it by adding distilled water and then determining the survival time of the residual red cells?

MOLLISON We haven't tried the interesting experiment you suggest, however L E Young and his co-workers have shown by a different method that young red cells are less resistant to hypotonic saline but are more resistant to mechanical trauma than old cells. C A Finch has some evidence that younger red cells are more readily damaged during storage than older cells

MERRILL I am very much interested in the phenomenon that has been mentioned, the sodium exchange with potassium, and particularly

the potassium loss on storage in the cold. That certainly is not due to a mechanical defect of the cell membrane, because the cell is freely permeable to potassium, and the differential concentration must be maintained by some energy-requiring mechanism, presumably through the adenosine triphosphate I wondered if one might get some clue as to whether this is a utilization of high-energy phosphate bonds to the point of exhaustion, or whether it is a failure to metabolize, perhaps due to the cold suppressing energy-producing mechanisms Have you made any attempts to measure the ATP?

MOLLISON Prof Maizels has recently made some measurements for us and has concluded that there is little change in easily hydrolysable phosphorus during storage

HARRIS With regard to the metabolism of cells at low temperature, Bovarnick and his colleagues (*J Bact*, 1950, 59, 509-522), who were trying to preserve *Rickettsiæ* at low temperatures, found that the addition of glutamate to the suspending medium allowed greater survival of the organisms after freezing to −20°C They suggested that this was because the glutamate was a substrate for the organism—which could of course mean that there was some metabolism going on even at that temperature

I believe you said you used trisodium citrate in your medium and lost potassium Have you tried potassium citrate?

MOLLISON My co-worker, Dr Chaplin, has tried storing red cells in potassium citrate and has shown that the loss of potassium from red cells is thereby considerably reduced However this does not have any appreciable effect on their survival *in vivo*.

BRENT You mentioned at the beginning of your talk, Dr. Mollison, that it is not possible to transfer leucocytes Could you give us a reason for this particular phenomenon?

MOLLISON I am afraid not. I was thinking of the work of Weisberger and others showing that if you transfuse white cells from one animal to another they are almost immediately trapped in the pulmonary circulation

BRENT Do you know of a method of freezing leucocytes for long-term storage?

MOLLISON Attempts to store white cells have been made by Tullis in Boston and I think by Dr Audrey Smith Perhaps she would say something about that

A U SMITH I haven't done a great deal so far, but I have found that leucocytes will keep as long as three weeks at +5° in fluid withdrawn from the peritoneal cavity of rabbits At the end of three weeks there were very large numbers of highly active leucocytes—that is to say, active *in vitro*, showing amœboid movement and active phagocytosis.

We have also been trying to preserve them at very low temperatures The first difficulty is in the administration of an adequate concentration of glycerol to protect them I am now able to get leucocytes actively moving in 7 5 per cent glycerol and 10 per cent glycerol, but so far I have not found a method of cooling them which enables me to keep them in a frozen state at very low temperatures

BILLINGHAM Have you attempted to preserve leucocytes at low temperatures after first compacting them into a more or less solid mass of cells, instead of preserving them in the form of a suspension? Prof Medawar and I have found that epidermal cells when dissociated in suspensions are much more susceptible to freezing and thawing than when they are in the epithelium of an intact graft.

A SMITH That has been exactly my experience I find that if I have a suspension of leucocytes they don't last nearly so well as if I get them into a compact pellet, so far only leucocytes kept in a solid mass have survived storage at +5°C for more than one week For reimplantation it might be useful to have pellets of leucocytes but for transfusion it would probably be useless

DEMPSTER Is it the lung that mops up the leucocytes? Why should that be?

MOLLISON I really don't know

DEMPSTER It has been shown that leucocytes are antigenic, as distinct from the blood red cells It is possible that the leucocytes act as foreign bodies and are knocked out just as bacteria would be knocked out

MOLLISON The rapid removal of transfused leucocytes from the circulation is probably not due to the action of antibodies, since it has been shown that leucocytes taken from an animal's own peritoneal cavity and injected intravenously are also rapidly removed from the circulation

EARLE To what extent, Dr Mollison, have you been able to rule out the injurious effect of light on the washed cells?

MOLLISON I doubt if light plays any part in producing the damage we observe since we have found that fresh red cells washed repeatedly in saline, in daylight, survive normally.

EARLE I think if you check that you will find that visible light will induce a later hæmolysis

MOLLISON Is it a slow cumulative effect over time? Our blood is stored in the dark but the cells are washed out without any special precautions

EARLE But if they have been subjected to light, I would not rule out the possibility that when they were later brought out and re-suspended, that the light had increased their susceptibility to lysis

PARKES Is the ordinary blood bank kept in the dark?

EARLE In the ordinary blood bank, the cells are in serum and are far less sensitive to the action of light Washed cells are extremely sensitive to visible light, including wavelength frequencies in the red, green and blue

MEDAWAR I wonder if Dr Mollison could explain why some rhesus negative volunteers when transfused with rhesus positive blood do not form antibodies

MOLLISON There appears to be great variation between different individuals If rhesus negative volunteers are given two intravenous injections of Rh positive blood at an interval of three or four months, only about 40 per cent of them will form rhesus antibody However if further injections of rhesus positive blood are given to the remainder, the proportion who form antibody steadily increases

MEDAWAR So there isn't any section of the population that is wholly refractory, so to speak—any group of rhesus negative people that never form rhesus antibodies?

MOLLISON There is insufficient experience for a dogmatic answer but it seems probable that all rhesus negative persons are capable of forming rhesus antibody For example, in one series, after six injections, nine out of ten volunteers formed antibody

MICHIE You mentioned intraperitoneal blood transfusions, with which animals and at what ages?

MOLLISON Both in man and in dogs it has been shown that red cells injected intraperitoneally reach the blood stream.

BRENT I think Dr. Mollison said earlier that one-twelfth of all transfusions showed some mysterious reaction and destruction of red corpuscles within sixty days Would you expect a slow disappearance of red cells if it were due to another undetected blood group? Would such destruction take place over a very long period of time?

MOLLISON In all known forms of incompatibility in man described so far, in which specific antibodies can be demonstrated, the red cells are eliminated within a few days of the first appearance of the antibody. However, L E Young has encountered a variety of incompatibility in the dog in which quite slow elimination occurred despite the presence of antibody In the particular case I am thinking of the transfused cells possessed what has been described as a low-grade antigen, comparable to the human antigen D^u.

BIOPHYSICAL ASPECTS OF THE FREEZING OF LIVING CELLS

J E LOVELOCK

THE investigator concerned with the adverse effects of low temperatures on living cells is confronted with three distinct problems Firstly, there are the destructive effects resulting from the crystallization of water within or without the cell Secondly, the adverse effects of cooling, thermal shock, which may occur with equal readiness above as well as below the freezing point Thirdly, the slow deterioration which takes place on prolonged storage even at temperatures as low as −190°C

In this short account it is not possible to do justice to more than one of these problems and for that reason I propose to deal only with the first, namely the adverse effects of freezing and thawing.

Most cells live in contact with a dilute aqueous medium and the effects of freezing and thawing are transmitted to them through this medium In order to appreciate the stress applied to a cell during freezing and thawing it is helpful to consider the processes which take place when a dilute aqueous solution is frozen and thawed If a test-tube containing one per cent sodium chloride solution is frozen by immersion in a low temperature bath, ice is seen to form as an opaque concentric shell which moves inwards until all the solution has frozen. If the freezing is arrested before the inward moving shell has filled the tube, and the shell of ice and unfrozen solution are separately analysed, it will be found that their bulk compositions are identical and equal to that of the original unfrozen solution Since ice separates from a salt solution as a pure substance this observation can only mean that the inward moving shell is not solid ice but a mesh of

ice crystals with concentrated salt solution in the interstices. If the conductivity of the mesh of ice crystals and salt solution is measured during freezing it is found that there is little change until all of the solution has frozen. This observation implies that the channels between the ice crystals are continuous throughout the mesh The continuity of these channels has been demonstrated rather more elegantly by Dr. H A. Sloviter (1951) in a personal communication. He showed that if a suspension of red blood-cells in glycerol-saline is frozen to −20°C and left for a few days the cells settle through the channels. The clear white supernatant and apparently solid mesh of ice crystals and the dark red sediment of blood cells illustrates strikingly the freedom of movement still available to the cells even at this low temperature.

It follows that when a suspension of living cells is frozen their first experience, apart from a drop in temperature, will be an increase in the concentration of their suspending medium. Crushing and spearing by ice crystals, if it occurs at all, can only come later when the channels between the ice crystals have shrunk to the same dimensions as those of the cells. The response of a given cell will depend in the first instance upon its permeability to water. If it is relatively impermeable—for example the fresh water amœba—its internal medium will remain unchanged and super-cooled as freezing progresses, eventually, however, usually in the region −5 to −10°C., it will freeze internally Chambers and Hale (1932) and Smith, Polge and Smiles (1951) have shown that this event is invariably fatal. Cells which are highly permeable to water—most mammalian tissue cells for example—will remain in osmotic equilibrium with their suspending medium as freezing progresses. That is to say, they will lose water during freezing at a rate which maintains the freezing point of their inner contents equal to that of the suspending medium. By the time the suspending medium has concentrated to saturation, at its eutectic, the cell will be too dry to freeze. Very few cells are capable of withstanding exposure to concentrated salt solutions, consequently most

cells are irreversibly damaged long before their suspending medium has concentrated to saturation In practice it is found that −5° represents the lower limit to which most mammalian cells can be frozen slowly without damage. At this temperature normal physiological suspending media would have concentrated nearly ten times. The destructive action of concentrated salt solutions, although rapid, is not instantaneous, and if the cells can be frozen rapidly they may be preserved for long periods at temperatures below −40° when all is solid. It follows that thawing must also be rapid for successful revival. To summarize—most cells are rapidly destroyed when frozen to temperatures between 0 and −40°C. With cells which are permeable to water, this critical region of temperature appears to correspond with a critical region of salt concentration. With these cells also no directly harmful results attributable to ice crystal formation appear to occur, such as have been suggested by Luyet (1949) and Strumia (1949) The demonstration of damage by ice crystals is likely to be very difficult since the cell would almost certainly have been destroyed by salt before the ice could harm it further

The nature of the destructive action of concentrated salt solutions is complex. The red blood-cells are the only tissue in which this feature of freezing damage has been subjected to systematic examination (Lovelock, 1953*a*) With these, sodium chloride solutions stronger than 0 8 M render the cell membrane cation-permeable Hæmolysis then takes place either slowly in the strong solution, or immediately when the cells are restored to isotonic media, as on thawing In very strong solutions, 2 0 M and higher, the structural integrity of the cell is destroyed. It is possible to account quantitatively for the hæmolysis which occurs when red blood-cells are frozen for various times at a given temperature in terms of exposure to the salt concentration in equilibrium with ice at that temperature No originality is claimed for the idea that the first adverse effect of freezing is the concentration of the electrolytes of the medium Moran (1929) showed that the

damage suffered by frog muscle on freezing could be explained quantitatively in terms of electrolyte concentration.

It was discovered by Polge, Smith and Parkes (1949) that living cells could be frozen and thawed without harm if glycerol were included in their suspending medium. This discovery is not only of great practical value in the successful preservation of tissue at low temperatures but also provides important experimental evidence relevant to the general problem of freezing and thawing.

The effect of including glycerol in the medium used to suspend cells is to reduce the range of critical temperatures in which damage takes place. Fig. 1 shows the hæmolysis due to freezing human red blood cells at temperatures between 0° and —40°C. for ten minutes. The cells were suspended in 0·9 per cent sodium chloride containing various concentrations of glycerol. It is evident from the figure that as the glycerol concentration increases so the area of the critical region decreases until finally at a concentration greater than 2·5 M it vanishes. Closely similar results are obtained if other cells, for example spermatozoa, are frozen under the same experimental conditions.

It has been proposed that the critical region of temperature corresponds to a region in which the salt concentration of the cell and its medium are above a critical value for the cell in question. If this proposition is accepted it provides a simple explanation of the action of glycerol, namely that it functions as a "salt buffer"

It follows from the colligative properties of solutions that the mere addition of a neutral solute to a salt solution will lower the concentration of salt in equilibrium with ice at any temperature below freezing. This may be expressed more precisely as follows· The depression (T) of the freezing point of an aqueous solution of sodium chloride and glycerol, the concentrations of which are x and y respectively, is given by the relationship $T=K(2x+y)$ where K is the freezing point depression of water produced by unit concentration of solute

If the critical salt concentration above which a cell is

irreversibly damaged is given by (nx) then the temperature (T_D) below zero at which irreversible damage will first occur is given by $T_D = Kn(2x+y)$ This equation can be transposed to give the glycerol concentration (y) required to prevent irreversible damage at a given temperature

$$y = \frac{T_D - 2Knx}{2Knx}$$

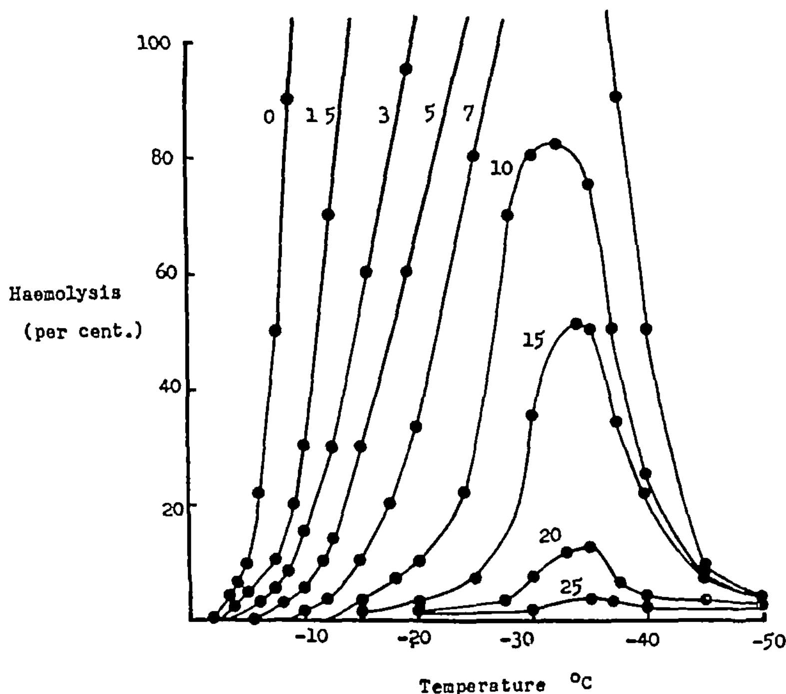


FIG 1 The hæmolysis which occurs when red blood-cells suspended in 0 9 per cent sodium chloride containing various concentrations of glycerol are frozen In all experiments the cells were frozen for ten minutes at the temperatures indicated

This relationship can be used to test the validity of the hypothesis that glycerol prevents damage during freezing and thawing by functioning as a salt buffer The concentration of glycerol required in practice to prevent damage can be compared with that required by theory Fig. 2 shows lines plotted according to the equation above and the experimentally observed concentrations of glycerol required to

prevent damage These results offer strong support for the proposed explanation of the function of glycerol

This relationship has already proved to be of practical use in suggesting that the recovery of red blood-cells preserved in bulk would be improved when the salt content of the suspending medium was reduced and the glycerol concentration increased before freezing (Chaplin and Mollison, 1953; Lovelock, 1953b) It may also be of use in suggesting favourable conditions for the preservation by freezing of a tissue which has not previously been subjected to successful preservation at low temperatures.

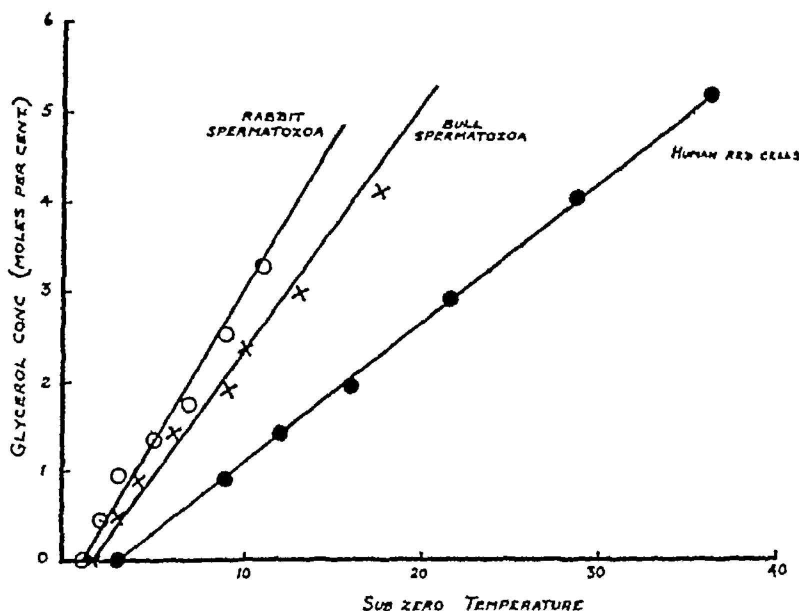


Fig 2 The concentrations of glycerol required to protect rabbit spermatozoa, bull spermatozoa, and human red blood-cells when frozen at various temperatures for ten minutes The lines are derived theoretically and the points are experimental observations

It is important to note that glycerol is able to protect against damage on freezing only when it has permeated the cells There is no benefit in preventing the salt content of the suspending medium from increasing during freezing if that of

the cell interior is allowed to rise unchecked This requirement explains why larger molecules than glycerol, for example the sugars, are rarely successful in preserving cells at low temperatures The desiderata for a substance which will protect living cells during freezing and thawing are as follows

(1) Low molecular weight.
(2) Miscibility with salt solutions
(3) Non-toxicity even in great excess
(4) Ability to permeate the cell freely.

With the red blood-cell a number of substances, including methanol, acetamide, and many polyhydric alcohols, are capable of meeting these requirements With more sensitive cells, e g sperm, none is quite so effective as glycerol

It is of interest to note that the protective activity of all substances tested was proportional to their molecular weight, as is required by theory

In this short account of the destructive effects of freezing and thawing and of the use of glycerol to prevent them, it has not been possible to discuss the important related phenomena of thermal shock and of slow deterioration on storage These effects and others render the successful preservation of living tissue much more difficult than the simple relationships described above might suggest (Parkes, 1953). Each advance in the physical interpretation of the adverse effects of freezing and of storage in the frozen state further reveals the magnitude of the task confronting the biologist who wishes to preserve living tissue, but pays high tribute to those who have already succeeded

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DISCUSSION

EARLE In the storage of fragile and critical solutions such as are used in tissue culture, can you suggest what is a safe temperature to avoid disintegration?

LOVELOCK That is a very difficult point. We find that even with cells each cell must be considered on its own merits There are some cells—for example, human sperm cells—which appear to tolerate a wide range of temperatures, and others—for example, the rabbit spermatozoa—which will not survive freezing to −2° or lower In general terms temperatures above −2° or below −40° are best for prolonged survival in the frozen state

MEDAWAR· Dr. Lovelock said that there was evidence of slow deterioration of tissues after storage even at −190°, but I don't remember what that evidence was Dr Billingham's and my experience in storage of skin is that there is no deterioration over a period of ten months.

PARKES In the media we have used, we have not yet managed to preserve fowl sperm without loss There are very few left after three months at −79° and after a year at −190°

MEDAWAR That would appear to be an exceptional case, as far as the empirical evidence goes for other tissues

S E SMITH I have a small amount of evidence from the anterior pituitary, which after storage for ninety days in liquid air has a very much greater delay before it begins to grow *in vitro* than after only a few days' storage, although it grows quite well eventually. I presume that means that a certain proportion of the cells have actually been lost during storage

PARKES I think that even if you contrive to freeze cells in liquid air and revive them without loss, that does not necessarily mean they will not suffer loss on storage.

PATE Lt Merriman in our Institution has shown that mammalian liver preserved at very low temperatures shows a steady increase in ice-crystal growth over very long periods of storage which will conceivably denature the protein and concentrate the salt

A U SMITH I should like to know how the ice crystal growth is measured at −190°?

PATE Essentially he took long tubes of dog liver, froze them, then sliced pieces at intervals and studied them microscopically (also with the electron microscope). I am not familiar with the details of the technique

HARRIS If I remember rightly it was done by a replica techniq Dr Merriman made a replica of the surface and studied that in electron microscope

PATE Yes, he did not have the direct tissue in the electron microsco

PARKES We have tried to interest crystallographers in this matt but current opinion seems to be that it is not a crystallographic proble

EASTCOTT What is the rate of penetration of glycerol into tissues different sizes and thicknesses? What sort of time should we allow, example, for arteries or slices of tumour tissue? Supposing we want bank a piece of human tissue under practical hospital conditions, h can we be sure that we shall apply the optimum conditions of glycer zation to these tissues before we freeze them?

LOVELOCK I'm afraid this again is a case of considering each tis on its merits The rate of penetration of glycerol, even with the blood cells of different species, shows enormous variation The red of the ox, for example, is only permeated by glycerol in a matter hours, whereas the human red cell is penetrated within a matter minutes This feature extends throughout the whole of natural tissu In dealing with tissues you not only have to consider the vary permeability of different cells but also the slow diffusion of glycerol i the relatively large bulk of tissue. I'm afraid as far as I can see it is j a matter of using successful preservation as a criterion of penetration

EASTCOTT But we have some sort of guarantee that glycerol is rat an innocuous substance So that it would be better to err on the l side rather than to attempt to freeze them too soon after putting th into the glycerol?

LOVELOCK Dr Smith has had more experience in dealing with tiss than I have Perhaps she would like to answer that.

A U SMITH The rat ovary survived some hours in glycerol, but the other hand twenty minutes' preliminary glycerol treatment of small fragments that I was using in the earlier experiments permit good survival on exposure to very low temperatures

EASTCOTT You have not in fact seen examples of glycerol damage?

A. U. SMITH Glycerol damages the eggs in the larger follicles but granulosa cells and the other endocrine components seem to toler glycerol well

PARKES We found that adrenal cortical tissue of the rat does not l being soaked overnight in glycerol I think there is probably a rat sharp optimal limit of time, but what it is we don't yet know

HUFNAGEL Dr Lovelock, are there any data available as to the creased survival of tissues if the temperature is allowed to vary wit rather minor limits below $-20°$ Say we have a variation betw $-20°$ and $-30°$, with a fluctuating temperature, does this affect surviv

LOVELOCK I haven't had direct experience of this, but whet glycerol is present or not, there is with most cells a peak death rat some given temperature With red cells it corresponds to $-35°$, with the bull spermatozoa about $-20°$ Clearly if the fluctuat temperature brings the cells close to that peak, then survival will affected adversely I think one could predict that

EASTCOTT: May I take it then, that under these circumstances there is no question of vitrification occurring or being facilitated by glycerol?

LOVELOCK: Whether or not vitrification occurs, it isn't really necessary to invoke it in order to explain the effects that we have observed. Also, Pryde and Jones (*Nature*, 1952, **170**, 685) have shown that the vitrification of water is particularly difficult to achieve, and that when it is achieved, water will only remain in a vitreous state at temperatures below −130°, and that it reverts almost instantly back to crystalline ice at temperatures above −130° Glycerol does not prevent the formation of ice crystals

PARKES This use of glycerol to protect the cell in freezing and thawing was originally worked out in a highly empirical fashion, and we had some 2½ years' start on Lovelock before he joined us to try to find out exactly what we were doing He has, as you have heard, disentangled a large part of the story, and we hope that any moment now he will pass from diagnosis of what we have been doing to prognosis of what we ought to do

METHODS FOR ANALYSING THERMAL STRESS WITH TISSUE CULTURE TECHNIQUE*

C. M. POMERAT and ROBERT B LEWIS

IN contrast to *in vivo* tests of the survival of tissues following their storage, cellular emigration from fragments explanted *in vitro* provides incontestable proof of the viability of various species of cells.

A great stimulus toward the improvement of tissue storage practices has resulted from mounting evidence that the theory of ice formation as the lethal factor in cold injury was false The brilliant work done in this country by investigators at the National Institute for Medical Research has brought clear proof of the importance of relatively slow cooling in an adequate medium, as represented by 15 per cent glycerol in blood serum, to ensure cell survival after exposure to very low temperatures.

The work to be reported was begun with quite different goals in a study of frostbite Lewis and Freytag (1952) had shown, by means of simultaneous potentiometric recordings from several thermocouples placed at various points in a rabbit's leg which was frozen under standard conditions, that the tibialis anticus cools more rapidly and reaches lower temperatures than other leg muscles Histopathological studies showed graded injury muscle and nerve being quite easily damaged, while skin and tendon were found to be considerably more resistant Our *in vitro* experiments were aimed at describing the relative critical injury levels for various tissues, with special reference to skeletal muscle. In the course of the study, several observations were made and tissue culture techniques were developed which it is hoped may serve others in the quest for the ideal conditions required in the preservation of normal organs

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General Methods

Procedures developed to test the viability of cells following rapid freezing and thawing have been described by Pomerat and Lewis (1953). These are summarized here together with the essential results which have been accumulated thus far.

Tissues to be studied are cut with sharp knives in squares approximately 1 mm. on each side. Human skin obtained for grafts should be as thin as possible if epithelium is to be encouraged. Spinal cord fragments from nine-day chick embryos, which are particularly useful for the study of emigrating nerve fibres, are cut in slices ⅛ to ¼ mm. in thickness (cf. Pomerat, 1951, p. 268) It is important to use very sharp knives and to avoid undue trauma in handling the tissues This is especially true for cultures of skin (Fig 1).

The viability of tissues with a high growth potential, such as that obtained from embryos and new-born individuals, can be determined most readily by means of the simple hanging drop culture technique. This has also been found particularly useful for the cultivation of epidermis (Lewis, Pomerat and Ezell, 1949) and the nasal mucosa of human adults (Rose, Pomerat and Danes, 1949); the critical factor in such preparations being that two or, better, four drops of a protein-containing nutrient fluid such as human serum or ascitic fluid be added on top of the plasma clot The essential steps in the execution of this method are shown diagrammatically in Fig. 1. It has proven especially valuable in a study of skin viability in relation to methods of storage (Allgöwer and Blocker, 1952), for observations on auto- and homograft survival (Allgower, Blocker and Engley, 1952), as well as the analysis of so-called "wound healing agents" (Allgöwer, Pomerat and Blocker, 1952) and the toxicity of antibiotics (Hu, Livingood, Johnson and Pomerat, 1953).

For most tissues of adult origin and especially for situations in which special nutrients or drugs are to be added to the culture medium, the roller tube method of Gey and Gey (1936) is particularly valuable. For the purpose of examining

cells in the living state with both still and cinematographic phase microscopy as well as for permanent stained mounts, it is advisable to prepare cultures on narrow cover glasses which are inserted freely within the roller tubes In Fig 2A the first step in this procedure is illustrated, while Fig 2B shows two cover glasses of No. 1 thinness measuring 12×50 mm being inserted "back to back" within a Pyrex test tube

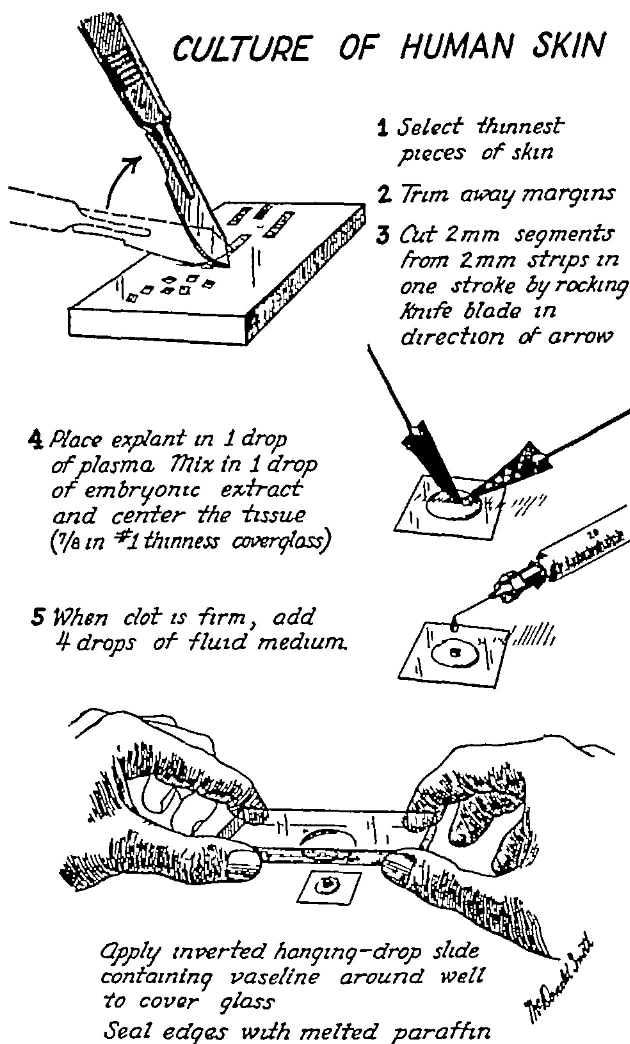


FIG 1 Technique for the cultivation of human skin in hanging drop slides

sequently thawed in a constant temperature bath (e) maintained at +42°C. Tissues are cultured following exposure to various temperature changes

The Effect of Rapid Freezing and Thawing

More than 1000 fragments of chick embryonic tissues and nearly 700 explants of human skin were used in a series of experiments to determine the effect of freezing to temperatures as low as −39 2°C within one minute, followed by rapid thawing Details of the results obtained have been published (Pomerat and Lewis, 1953), but for the purpose of the present report Figs. 4 to 10 summarize the essential findings obtained to date. Using tissues from eleven to thirteen day chick embryos, emigration of multinuclear skeletal muscle fibres was obtained after exposure to approximately −20°C (Fig 4), of epithelium after −30°C. (Fig 5) and of spindle cells at about −40°C (Fig 6) Human preputial skin showed epithelial survival after −24 4°C. (Fig 7) and spindle cells survived after exposure to −29 2° C. (Fig. 9) Explants of adult human epidermis gave rise to epithelial sheets after exposure to −20 5°C (Fig 8), and spindle cells after −29 2°C (Fig 10)

It appears, therefore, that under identical experimental conditions chick embryonic epithelial and spindle cells were somewhat more resistant to rapid freezing and cooling than corresponding cell types from human skin Little difference was found between new-born and adult human tissues with respect to their vulnerability to cold injury. Spindle cells from the chick and human skin were more resistant than those cultivated from leg muscle fragments obtained from the corresponding chick embryos In several instances when spindle cell out-growth was limited to only two or three cells, these could be shown to have arisen from the area of blood vessels in the explant An example of this phenomenon is shown in Fig. 11, which was obtained after freezing twelve-day chick leg muscle to −35 4°C. in twenty-five seconds It is not believed that these cells were derived from

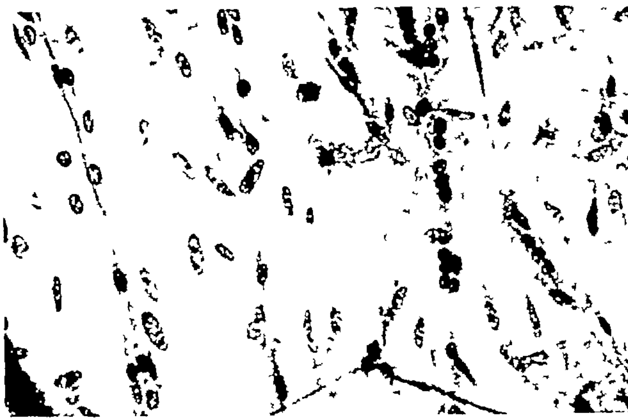


FIG 4 Muscle −20 6°C in 29 sec

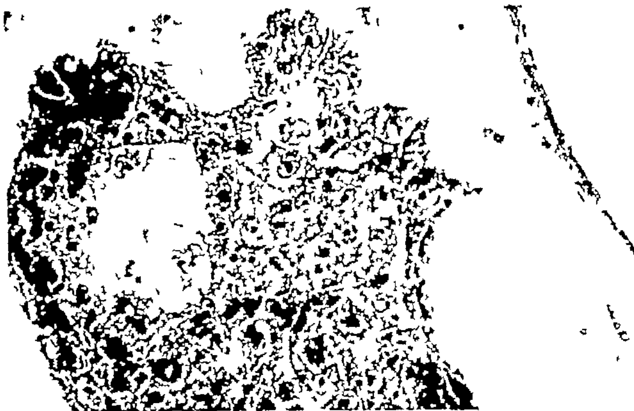


FIG 5 Epithelium −29 8°C in 43 sec

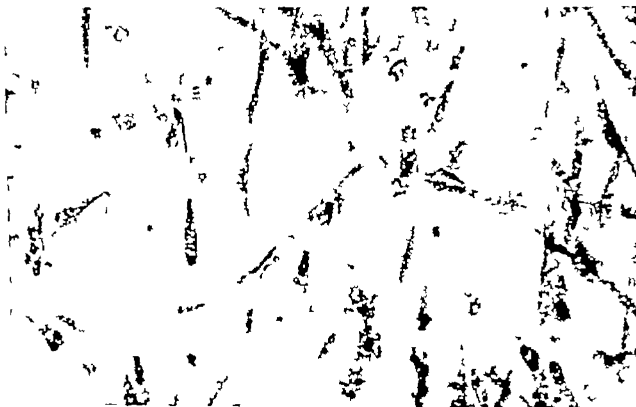


FIG 6 Spindle Cells −39 2°C in 48 sec

FIGS 4–6 Photomicrographs of stained tissue culture preparations showing the lowest temperature achieved following which muscle, epithelial and spindle cells successfully emigrated from explants of thirteen-day chick embryos Magnification approximately ×400

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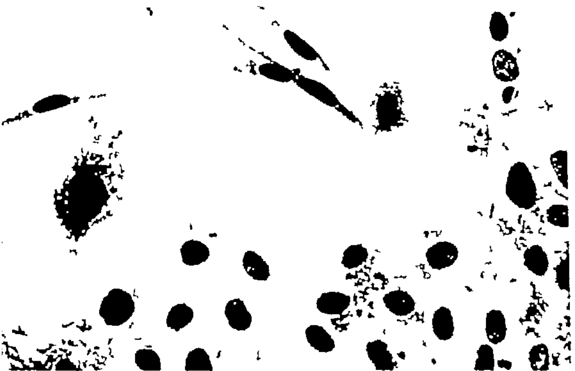


Fig 7 Epithelium −24 4°C in 40 sec

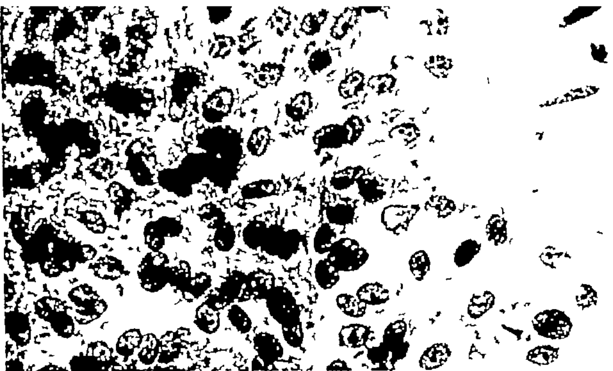


Fig 8 Epithelium −20 5°C in 35 sec



Fig 9 Spindle Cells −20 2°C in 53 sec



Fig 10 Spindle Cells −20 2°C in 53 sec

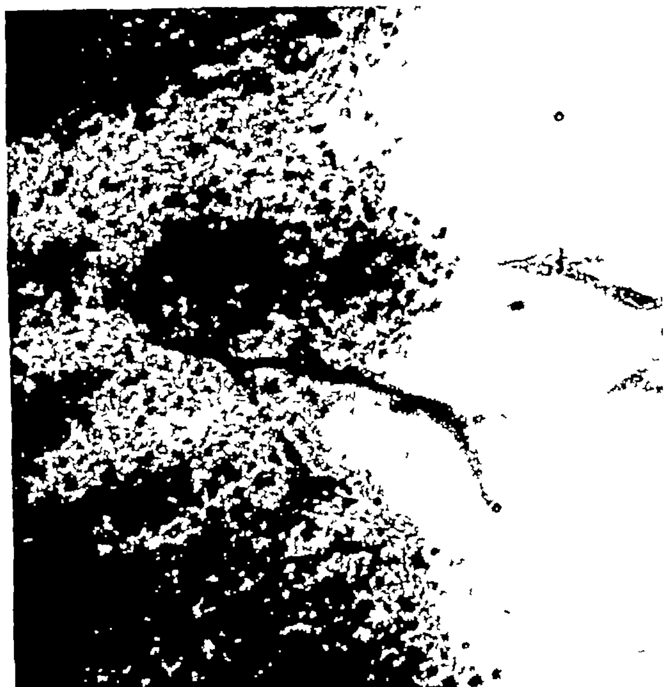


FIG 11 Spindle cells which emigrated from vascular structures of an explant of twelve-day chick leg muscle after exposure to $-35.4°C$ produced in 25 seconds Such cells appear to be the elements which are the most resistant to cold injury Magnification approximately ×800.



the endothelium but that they probably represent perivascular elements of a primitive type Thus, it would seem that our results lend additional support to the concept of "physiological races" of spindle cells to which Raymond Parker (1933) contributed the significant observation that characteristic growth rates *in vitro* are obtained for strains of fibroblasts derived from various organs Lewis (1951) also observed that during healing of necrotic frostbitten rabbit leg muscle the fibroblastic growth can first be detected in the vicinity of blood vessels

Work in progress is concerned with a comparison of critical thermal injury to other species of tissues and organs which can easily be managed with the present technique.

Rapid versus Slow Freezing

Characteristic results in this series are illustrated by two experiments (Nos 65 and 66) in which twelve-day chick tissues were frozen to approximately the same level in sixty seconds, as against slightly more than five minutes Twenty cultures were used for each experimental condition These were stained by Jacobson's method (Giemsa—May—Grunwald) after six days' incubation and the following observations were made

	Leg Muscle	*Skin*
Experiment 66 Freeze to −20°C in 60 sec	Muscle and spindle cells in 8 Scant spindle cells only in 5 No outgrowth in 7	Epithelium and spindle cells in 4 Scant spindle cells only in 15 No outgrowth in 1
Experiment 65 Freeze to −19 8°C in 320 sec	Muscle and spindle cells in 4 Spindle cells only in 16 Spindle cells less dense than corresponding controls	Epithelium and spindle cells in 8 Spindle cells only in 17 Spindle cells somewhat less dense than those of controls
CONTROLS	Muscle and a rich outgrowth of spindle fibres in all cultures	Epithelium and numerous spindle cells in all cultures

Rapid freezing certainly reduces the total amount of outgrowth but it is interesting to note, especially in this series of experiments, that whenever the emigration of spindle cells, presumably of the fibroblastic type, is inhibited muscle spindles and epithelia are encouraged. This result did not appear to be due to differences in *p*H but possibly to competition for nutrients in the immediate vicinity of the explants.

It will be noted that with the use of the potentiometric recording technique, super-cooling effects were obtained, which were characterized by a sudden rise in temperature soon after the zero point had been passed when a bath at approximately −20°C. was employed for the freezing operation. This phenomenon has been observed in the course of *in vivo* experiments by Lewis and Freytag (1952). Whether or not its occurrence in freezing procedures is important in relation to recovery from the injury has not yet been determined in the present series of experiments.

The Intensity-Time Factor

Recent work (Lewis, 1952) has served to emphasize that several days' exposure to temperatures of as high as +60° to +70°F. reported in cases of "immersion leg" can produce gangrene identical with that found in frostbite. This indicates the importance of the intensity-time product in thermal injury to cells

While freezing of tissue fragments in balanced salt solution is admittedly less physiological than that achieved in the 15 per cent glycerol-serum mixture used by A. U Smith (1952), there is evidence in our experiments of greater injury to cells exposed for thirty minutes than for a period of five or less minutes Under these conditions no outgrowth was obtained in only one out of 20 cultures of muscle for the two experiments with periods of short exposure, but 14 out of 20 cultures were totally inhibited in the thirty-minute series. With one-half hour exposure, muscle fibres were completely eliminated and spindle cells were markedly reduced and showed vacuolation. In contrast to luxuriant cultures in the

controls and for the short exposure, skin frozen for five minutes produced epithelium in 6 out of 20, scanty spindle cells and no outgrowth in 4 out of 20 cultures. For skin frozen for thirty minutes, no outgrowth was obtained in 9 out of 20 cultures, spindle cells were markedly reduced and no epithelial elements emigrated

Efforts to Relieve Thermal Stress

The technique of imposing cold stress on tissues under standard conditions with accompanying potentiometric records of the induced thermal changes also suggests the opportunity to use tissue culture methods in the quest for agents to overcome physicochemical disturbances, to neutralize the effect of toxic bodies which may be liberated as a consequence of the injury, and to provide additional nutrient factors which might enhance the course of the tissue recovery

In addition to the work cited (Smith, 1952), Luyet and Gonzales (1951) found protective action from 30 per cent ethylene glycol used as a dehydrating agent for chick embryo heart fragments frozen in liquid nitrogen and subsequently cultured *in vitro* In preliminary work we found that cultures of thirteen-day chick leg muscle and skin frozen to −34·2°C. in one hundred and forty-three seconds in 30 per cent glycerol in Gey's balanced salt solution produced muscle fibres, epithelium and spindle cells resembling those of controls which had been allowed to stand at room temperature for the same period in the glycerol-salt solution mixture In contrast, skin frozen to −32°C in ninety-seven seconds showed no epithelium and spindle cells in only one out of 20 cultures. While the time required for freezing was not identical, the protective merit of glycerol even in combination with balanced salt solution appeared certain in this experiment

In an effort to determine whether the mechanism of glycerol protection was primarily associated with its rôle in increasing the osmotic pressure and in relation to other goals described in the next section, glucose at a final concentration of as high as 1 2 g per cent was incorporated in the medium used as the

fluid phase for chick muscle frozen to −27 0°C. (in eighty-eight sec.) and skin frozen to −26·7°C. (in one hundred and seventy-two sec.) and to −32°C. (in ninety-seven sec). Following six days' incubation in the presence of this high concentration of glucose, the cells showed little evidence of injury, but no relief of the damage seen in untreated frozen control cultures.

In keeping with current views on stress phenomena, tissue injury due to cold might be expected to incite the mechanism of this reaction. In an effort to determine whether the balanced salt solution contained toxic bodies following freezing to −22°C. for one and a half hours, 1 ml. of supernatant fluid obtained after centrifugation of 30 fragments in a 2 ml. aliquot was used to constitute 50 per cent of the fluid for the nutrient used on three-day-old unfrozen cultures of twelve-day chick leg muscle. Suitable controls were prepared with balanced salt solution which had had contact with a similar number of tissue fragments for the same period of time but without exposure to freezing, as well as controls containing 1 ml. of balanced salt solution. After four days' incubation, cultures were fixed and stained. Microscopic examination revealed no significant differences in the four types of preparations

In studies directed at exploring the mechanism of allergic reactions with tissue culture methods, efforts have been made to describe the toxicity of several relevant chemicals on the basis of short-term contact with suitable cells Advantage was taken of this information in designing experiments which might shed additional data on the pathology of cold injury.

In hanging drop preparations of epithelium from human skin tested with histamine dichloride, total inhibition was found in the presence of 4165 μg /ml., while some cellular emigration resulted with 2080 μg /ml. of this substance in the culture medium. With 830 μg./ml., there was slight injury to the cells, while 415 μg./ml. did not appear toxic as compared to the corresponding controls. These values may be of interest

in considerations of possible histamine release as a factor in cold injury.

The antihistaminic Benadryl has been shown to inhibit epidermal outgrowth at a concentration of 330 μg./ml., but a few cells emigrated at 165 μg./ml. While some damage was found in the presence of 40μg./ml., contact with 30 μg. Benadryl/ml. for seven days resulted in no limitation of outgrowth, although cells appeared to form more "compact" sheets even with as little as 15 μg /ml

The toxicity of various cortisone preparations on chick tissues *in vitro* has been described by Ruskin, Pomerat and Ruskin (1951). No injury to the outgrowth of human skin cultures was found after seven days' contact with 200 μg./ml of cortisone tricarballylate.

Results from a recent experiment illustrate a procedure being used as a fresh approach to frostbite therapy which might also prove useful in dealing with the problem which concerns this Symposium.

Thirteen-day chick embryonic muscle was frozen to −27°C. (in eighty-eight sec) and the corresponding skin to both −26·7°C. (in one hundred and seventy-two sec) and to −82°C (in ninety-seven sec.) Eighty fragments were used for each experimental condition and 20 pieces were cultured without exposure to cold (unfrozen controls) Immediately after thawing, the frozen tissues were set up in roller tube cultures. A basic nutrient was used throughout. A control group received no further treatment, but 20 fragments in each of the three temperature series for both tissue species received an additional substance (1) glucose to make a total of 1 2 g. per cent, (2) Benadryl at 20 μg /ml , and (3) cortisone at 15 μg./ml Excess glucose, which is believed to be valuable in synthetic culture media (Waymouth, 1953), appeared to have little or no effect; the Benadryl was slightly damaging, but the cortisone appeared to exert some beneficial action, especially upon the spindle cells in skin cultures frozen to −82°C., where 8 out of 20 cultures showed spindle cells as compared to only one positive culture in the

untreated frozen controls Moreover, outgrowth in the presence of cortisone was relatively heavy in 8 of the 8 cultures. In earlier experiments at a lower temperature (−18 2°C in thirty-four sec.), 10 μg/ml. of cortisone was found to produce a richer population of multinuclear straps from explants of eleven-day muscle These observations, while qualitative and only suggestive, warrant further investigation. Details of these experiments will be published elsewhere.

Attention is called to the finding by Nowinski and DeRobertis (1948) that the Q_{O_2} of guinea-pig liver slices dropped from an average value of 6 47 to 2 04 following freezing in liquid air (−90°C) and quick thawing. The respiration of such material could be restored to the normal value (6·38) with the addition of M/50 sodium succinate. It was concluded that low temperature injury involved damage to the cell membrane resulting in the loss of this substrate. This direction of exploration invites further study

Cold and the Cell Organoids

For some years we have been using a perfusion chamber to study the effect of changing the chemical environment of cells in tissue culture while recording their activities with phase-contrast time-lapse cinematography * In the course of a programme of investigations on the biology of the human nasal mucosa, nuclei were occasionally seen to rotate in both clockwise and counter-clockwise directions One nucleus made 15 complete revolutions in seventy minutes, turning through 360° in seventy-five seconds at one point during this period (Pomerat, 1958). In order to determine whether this phenomenon accompanied sudden changes in temperature, a microthermocouple was introduced within the perfusion chamber (Figs 12 and 13) so that thermal changes could be correlated with events seen in the film record. While the initiation of nuclear rotation could not be clearly attributed to variations in temperature, at +37°C. the cells in a single

*A film on "The reaction of living cells to thermal stress" was shown

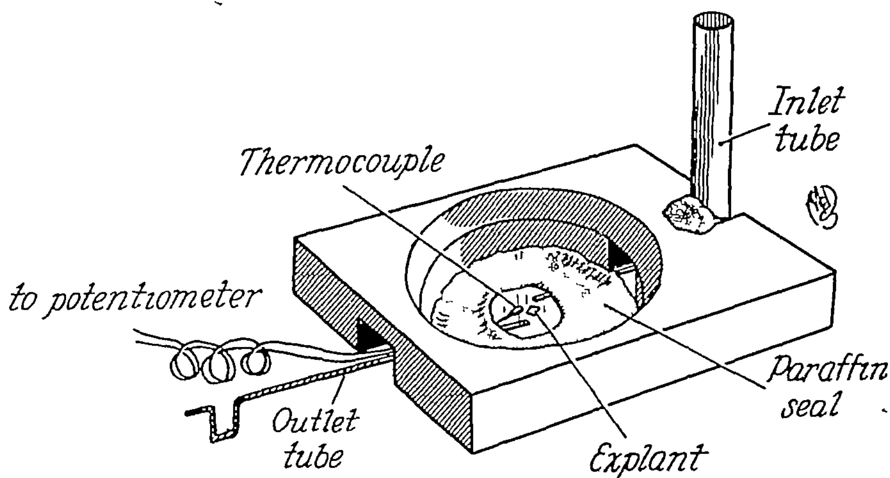


FIG 12 Perfusion apparatus used in making phase-contrast "still" and cinematographic records A thermocouple next to the explanted tissue is used to correlate temperature changes observed with a potentiometer Slightly less than actual size

FIG 13 Surface view at low power of a sheet of living human nasal mucosa (upper left) and a portion of the microthermocouple used in the apparatus shown in Fig 12 Magnification approximately ×100

[To face page 152

layer of mucosal epithelium were seen to undergo constant shifts in relative position. After dropping the temperature to +27 5°C this activity disappeared but was immediately reinitiated with a return to body temperature While such changes in activity are to be expected, the importance of these observations lies in the opportunities available for the exploration of thermal effects upon cell organoids such as

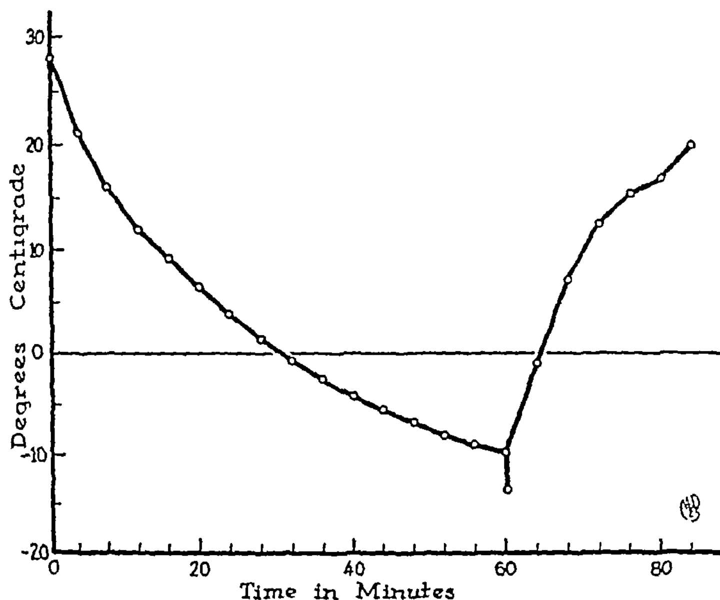


FIG 14 Potentiometric record of temperature changes in a perfusion chamber containing a four-day culture of adult human nasal mucosa in the presence of a fluid medium consisting of undiluted human ascitic fluid Photographs of the cells before and after freezing are shown in Figs 15 and 16, respectively

mitochondria with the highly controllable conditions provided by this instrumentation.

Fig. 14 shows the course of temperature changes from a thermocouple within a perfusion chamber containing a four-day culture of adult human nasal mucosa in a plasma clot but with a fluid phase consisting of undiluted human ascitic fluid. The pattern of intracellular structures with phase photomicrography was established immediately before (Fig 15) and after (Fig 16) the perfusion chamber was

placed in a deep freeze unit. Mitochondria were seen to become rounded and swollen Wrinkling of the nuclear membrane and increase in optical density suggested serious injury. No evidence of viability could be found upon subsequent observation. The experiment was repeated on the next day using similar culture material but with ascitic fluid containing 15 per cent glycerol Initial photomicrographs showed considerable cell shrinkage, as evidenced by lines of tension, cytoplasmic retraction processes (Figs 17 and 19) and by general surface ruffling (Fig. 18). The latter could be distinguished from mitochondria (Fig. 19) by careful focusing. A portion of one cell before (Fig. 19) and after (Fig. 20) exposure to cold did not show injury to the mitochondria in the presence of the glycerol. Cinematographic records confirmed these results.

Citing Matruchot and Molliard (1902) and Genevès (1951, 1952) in a recent review dealing with the morphological changes in plant mitochondria, Buvat (1953) has emphasized the importance of water loss from protoplasm as a result of low temperature. The formation of free water in the cytoplasm is believed to account for hypertrophy and vesiculation of the mitochondria, a phenomenon which is reversible under some conditions. It would appear that glycerol may prevent the formation of free water in the cytoplasm and thereby protect cell organoids.

Grateful acknowledgement is made for indispensable aid with tissue culture procedures to Mrs John Finerty and Mrs Frank Dunton, to Mr. George Lefeber and Mr Curtis Hoskins for photomicrographic records and to Mr. McDonald Smith for aid in the preparation of the graphs and diagrams.

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Figs 17 and 18 Five-day culture of epithelial cells in the presence of ascitic fluid containing 15 per cent glycerol, showing retraction and tension lines (Fig 17) and surface ruffling (Fig 18) before freezing

FIGS. 19 and 20. The processes of an epithelial cell from the human nasal mucosa showing the arrangement of filamentous mitochondria before (Fig. 19) and after (Fig. 20) exposure to freezing in a medium consisting of 15 per cent glycerol in ascitic fluid.

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DISCUSSION

EVANS Dr Pomerat, in your film you were associating the nuclear rotation with the presence of 2 nucleoli Do you suspect that nuclear rotation is more common in epithelial cells than in other cells?

POMERAT Yes. I have never seen the phenomenon except in epithelial cells I have never seen a fibroblast show nuclear rotation, although the classical fibroblast has a double nucleus, at least according to the textbooks of histology and in our own observations

EICHWALD Since it is unlikely that the nucleus rotates as a whole at the beginning, which part of the nucleus do you think begins rotating and drags the rest after it?

POMERAT I believe the whole nucleus rotates from the beginning When a notch in the nuclear membrane develops, we can trace that notch and see (especially when we reverse the film) that the whole thing is moving in an area where there is apparently quite a different viscosity I think the electron microscopists find a zone, perhaps not relatively quite as wide, close to the nuclear membrane, of quite a different density, and perhaps having distinctly different properties

EVANS Is it associated at all with the age of the culture?

POMERAT These are all quite young cultures, incubated for only five or six days

ROGERS Is it possible that extranuclear or cytoplasmic activity pushes the nucleus around?

POMERAT Some investigators believe that there are charges built up on the membrane, but there is little factual information available.

ROGERS In your pictures where there is an inflowing from the cytoplasmic membrane of vacuoles, all seem to be flowing toward the centre. It almost appears as if there is some definite, specialized activity of the cytoplasmic structures around the rotating nucleus

POMERAT One typically finds that there is a cell centre, whether you call it the Golgi apparatus, or the juxtanuclear area or a "zone actif". Very often we see activities proceeding to or from an area, and particularly in secretory cells we see a lot of movement at such a site It is possible that the nucleus has turned over and it is pivoting on this zone, or perhaps the polarization is not present at that moment, but we don't see any particular "centre" in the cytoplasm during rotation By plotting the nuclear position very carefully we had hoped to find that every time the nucleus came opposite a particular point in the cytoplasm there might be a change in rate, but the curves relating rotation to time are quite smooth There is no evidence of polarity as far as the cytoplasm is concerned

EARLE Have you tried to follow this rotation of the nucleus at any other frequencies of time-lapse photography?

POMERAT We have done it at one frame per minute and at eight frames per minute for different types of analyses Those that I showed were one frame per minute

EARLE In watching the pictures I got the feeling that there was some type of cyclosis in the cytoplasm of the cells pushing the nucleus around, but that it was possibly obscured by the frequency of the time-lapse photography, and I should like to see similar pictures at intervals of, say, 20 frames per minute.

POMERAT The nucleus sometimes moves a little laterally as it turns

EARLE If, for instance, you had a nucleus with a very heavy and large nucleolus, and if that nucleus were being pushed around centrifugally, it is very unlikely that it would rotate without some lateral shift also

A U. SMITH Did you get any photographs at the time of freezing to see what was going on in the cell and surrounding medium?

POMERAT No I should have explained the conditions under which the film showing freezing of cells was made We took the whole microscope out of the warm chamber and simply put it in an adjacent deep-freeze unit. We had expected that we should have all sorts of fogging of the lenses, and there is some, but in the still pictures there was no difficulty until water condensation in the interior of the tube finally gathered and went down to the objective You can do at least the preliminary screening that way. But we didn't follow it at the moment of freezing to discover whether we could see crystals or anything of that sort, although I think it is feasible

A U SMITH Mr. Smiles and I have recently been trying to find out

whether intracellular crystallization takes place in glycerol-treated tissues at very low temperatures We are using guinea-pig testis, which contains very large multinucleated spermatids Whilst cooling tissue treated with 15 per cent glycerol, the medium freezes, but in spite of surrounding ice crystals, many cells remain clear even at −60°C In absence of glycerol, intracellular crystallization invariably occurs between −5° and −10°C , and the cells then black out After thawing, tissue which has not been pretreated with glycerol is completely disrupted, whereas the glycerol-treated tissue is morphologically normal and the tails of some developing and fully developed spermatozoa are still moving when the preparations are re-warmed to body temperature

Similar experiments have been carried out with corneal tissue from the bull and the rabbit, but we had no obvious criterion of whether the cells were alive after freezing, and so we are continuing these studies with testicular tissue

[The illustrations shown as lantern slides will be published in due course in the *Journal of the Royal Microscopical Society*]

POMERAT We hope sometime to be able to follow such events with cinematographic records

PARKES The technical difficulties of doing photomicrography at −60°C are quite unbelievable

EARLE How long can these testis cells be maintained in glycerol at regular incubator temperature? Indefinitely?

A U SMITH We have not kept the guinea-pig testis tissue for longer than about an hour at incubator temperature but it was all right at the end of an hour

EARLE Is there any cell type that has been maintained for standard intervals in glycerol at incubator temperature?

PARKES You are thinking of some long period, are you? Days or weeks?

EARLE Yes

A U SMITH We have not kept anything in glycerol for more than a few hours at body temperature

POMERAT Our first idea about the glycerol effect was that we had a mild hypertonic reaction represented by cell shrinkage So we tried to imitate this with 1 2 per cent glucose and although the survivals of these cultures was not significantly different from the controls, there was absolutely no protection to cold The effect of glycerol is more than a mere matter of protecting against the hypertonic state As I mentioned, some plant cytologists have concluded that with cold, bound water is freed and the total amount of water that appears in the intracellular situation is responsible for the change in mitochondrial form It has been known for a long time that mitochondria, for example in the liver of *Amphiuma*, treated with hypertonic solution become long filaments, but with a hypotonic solution swell and tend to become spherical The formation of free water might be responsible for the differences in freezing behaviour of different species of tissue cells And perhaps the effect of glycerol is to protect against this—it dehydrates the cell a little, then some water comes out during the freezing

process and it equilibrates the system again We shall have to learn more about how much glycerol is needed for particular cell species in terms of the effect on the mitochondria

HARRIS. How do you interpret the cytoplasmic bubbling as you perfuse away the glycerol?

POMERAT My interpretation would be that the cell is suddenly taking on an enormous quantity of water. I suspect that the return to the isotonic state may be a very critical matter, at least with some cells Perhaps we should restore this gradually Certainly in the movie and in some still studies that we have done, there is a monumental amount of bubbling and blistering with the perfusion of the regular nutrient medium which is ordinarily employed for maintaining these cells in a healthy state

HARRIS Do you think then that the normal bubbling which takes place in some cells during division is due to the same thing—the cells taking on water?

EARLE That's exactly the question I was going to ask As far as I could see this bubbling was identical with the bubbling that occurs at the surface of a cell in certain stages of mitosis

POMERAT Yes, we see that regularly in mitosis, and of course it is also seen under various conditions of injury For example, in slight injury of leucocytes with radioactive substances, this bubbling on the surface has been described

MEDAWAR Is there any nucleated cell type in which pinocytosis does not occur? Is this the first demonstration of it in epithelial cells?

POMERAT No, I think that it occurs in most types of cells Dr. Arthur Hughes has demonstrated beautiful pinocytosis in the growth cones from nerve fibres cultivated from ganglia We see it in many species of cells, in fibroblasts it can be very common The mechanism regulating pinocytosis is a matter that needs much further study, apparently the presence of electrolytes is vital in determining its intensity.

MEDAWAR What sort of sense does it make of biophysical theories of cell permeability?

POMERAT Many of us in the tissue culture field feel that the cell doesn't give a damn for pretty drawings of interlocking proteins! The problem of getting something through that membrane is not at all a concern to the cell, when it can simply throw out a loop-like pseudopodium and then ingest a perfectly tremendous globule These globules aggregate and become quite large and then they move towards the indented side of the nucleus, and reduce progressively or suddenly disappear If there are large proteidial aggregates moving inside, then following the expulsion of the water it is possible that they are in direct contact with the enzymes of the cytoplasm The arrangement of molecules forming a "membrane" may have been present during the process of ingression, and surrounding the vacuole, but once that interface is destroyed, these bodies are quite free and in direct contact with the pattern of molecules or enzymes in cytoplasm Probably in this way many phenomena can be accounted for that do not yield to theories of mosaic patterns for the structure of the cell membrane.

HARRIS That does depend, of course, on whether you have a dynamic or a static concept of the cell membrane

POMERAT In any case these observations with phase cinematography of pinocytosis widen our horizon and give us more with which to work, whether we have a dynamic or static concept of the membrane

EARLE I should like to carry this line of thought on pinocytosis one step further When these droplets are taken in, the cells are living in a protein-rich solution Obviously, when the droplet is taken in, it must contain the protein Let us consider one of our strains of cells that has been in tissue culture for eleven years Let us conceive that a large part of the protein that is taken in by the droplet is pre-digested before it ever gets really into intimate contact with the actual cytoplasm of the cell Is it quite credible to assume that within that eleven-year interval *none* of that foreign protein ever really gets into the cell, in view of this pinocytosis? If the foreign protein does get into the cell, how is it going to affect the milieu in which the chromosomes and genes must operate? Even if you have potentially unchanging chromosomes and genes, are they going to operate in an unchanging manner in what may well be an altered milieu?

THE STORAGE OF SKIN

R E BILLINGHAM

THE storage of skin grafts has long been recognized to be a clinical problem of some importance, despite the fact that the fate of skin homografts at present practically restricts the application of stored skin to the individual from which it was removed initially In an emergency, of course, as a life-saving expedient, stored skin offers an immediately available source of homografts for use in providing temporary skin coverage Skin storage may sometimes save a patient the necessity of having to undergo repeated operations, since a sufficient supply to cover both his present and future requirements may be obtained at one operation and "banked". Experimentally, a reliable method for the long-term preservation of skin in a viable state is becoming increasingly desirable for a variety of purposes.

Skin is particularly suitable for studies designed to determine the optimum conditions requisite for tissue preservation with minimal damage to various cellular and fibrous components, grafts may be removed with ease, subjected to various treatments *in vitro*, and then grafted back orthotopically to the animal from which they were taken initially as a biological test of their continued viability Skin so grafted can be appraised for the rate at which epithelium grows out from its margins, the conservation of the pigmentation of its hairs and of its superficial epidermis, the normality of the hair crop which regenerates, the survival of its glands, the extent to which its fibrous organization remains intact, *etc.* In view of the existence of these numerous criteria, there can surely be little doubt that the grafting method offers a simpler and more accurate method of appraising the aggregate effect of an *in vitro* treatment on skin than does the *in vitro*

culture of small explants Grafting will establish not merely cell survival, but the survival of skin as an organized functional tissue

Storage at Ice-Box Temperatures

It is generally accepted that intact skin is a tissue highly resistant to thermal and other stresses; this is not unexpected in view of its exposed position in the body Skin also manifests its resistant qualities *in vitro* to a high degree, for example Pepper (1953) has shown that the epithelium of grafts of rabbit's skin which have been placed on damp blotting paper and maintained at 37°C. survives for three to four days, and at 0°C for about five weeks At intermediate temperatures intermediate results were obtained

As long ago as 1912 Carrel laid the foundations of what have now become the generally employed clinical methods of skin storage He demonstrated that flaps of dog's skin after being sealed off in tubes containing liquid paraffin as a preservative medium and stored at ice-box temperatures (between −1° and +7°C) "took" as well as fresh skin on grafting He also reported that skin removed from a dead infant and preserved in this manner healed into place as well as normal skin on homotransplantation. Ringer's solution he found to be inferior to petrolatum as a preservative medium The various clinical procedures now to be described are essentially slight modifications of Carrel's technique Webster (1944) in America reported a procedure which he had successfully employed on 23 patients over a period of years. On removal the grafts were cemented to sheets of pliofilm and then folded with their raw surfaces in contact to prevent evaporation Thus folded, the grafts were wrapped in additional pliofilm, after which the package was wrapped in several layers of xeroform or vaseline gauze to seal the grafts A final wrapping of two sterile towels was then added and storage carried out at 4°C It was reported that all the grafts survived autologous transplantation, provided that the recipient areas were suitable and the grafts had not been

preserved for longer than three weeks. Longer preservation, with one exception in which skin preserved for thirty-five days was grafted successfully, gave failures. Subsequently, Matthews (1945) in Britain described a series of successful clinical results obtained with skin stored for periods up to eight weeks—the longest period of storage required—with an essentially similar technique. The grafts, after being folded so that the raw surfaces were approximated, were wrapped in tulle gras followed by gauze tightly wrung out after soaking in saline. The packages were then placed in tightly stoppered bottles of 20 ml. capacity, care being taken to prevent the package containing the graft coming into contact with fluid collecting at the bottom. The storage temperature adopted was 3–6°C. Flatt (1948) made use of a similar method, the grafts being wrapped in tulle gras as before, but without the addition of the saline gauze. He maintained the skin at 32° –34°F. and reported high percentage takes with skin grafts transplanted within the first three weeks of storage, though even with longer storage satisfactory "takes" were obtained. Grafts from skin stored for as long as sixty-one days yielded 82 per cent "takes"

Subsequent to the appearance of these clinical reports of the satisfactory use of stored skin, the determination of the optimum conditions for the preservation of skin within this temperature range has been made the object of several experimental investigations. Hanks and Wallace (1949) carried out extensive tests on rabbit's skin stored either in 10 per cent serum in balanced saline solution or in mineral oil at 0°C. and at 6–8°C., the viability of the stored tissue being assessed on the basis of the *in vitro* cultivation of sample explants. They reported that exclusion of air was detrimental with storage at 6–8°C, viability declining within a week. This is surprising, since Medawar (1947) had previously shown that rabbit's skin epithelium can survive for upwards of a week in serum at body temperature under strictly anærobic conditions At 6–8°C. better results were obtained by Hanks and Wallace when their grafts were just covered with the storage medium.

As a storage medium they demonstrated that 10 per cent serum was superior to mineral oil. Storage at 0°C., they stated, gave less satisfactory results, particularly in the presence of air. The superiority of 10 per cent serum as the storage medium was explained on the grounds that it provides a nutrient and dilutes or buffers the acids that result from metabolism Both on the basis of the results of *in vitro* cultivation and on the results of grafting experiments, using rabbit's skin, Marrangoni (1950) has presented confirmatory evidence of the superiority of 10 per cent serum as a storage menstruum More recently Allgower and Blocker (1952) have described the results of their detailed study of the viability of human skin in relation to methods of storage. On the basis of tissue culture experiments they have reported that the methods of Webster (1944) and of Matthews (1945) are far from being optimal. They emphasize the marked superiority in viability obtained from human skin stored in 10 per cent serum in balanced saline at 5°C in the presence of air, over that obtained from skin stored in either vaseline gauze or wet (i.e. saline-moistened) gauze at this temperature Epithelial outgrowth *in vitro* was obtained from skin which had been stored in dilute serum for as long as twenty-eight days, whereas demonstrable viability in tissue culture was lost after twelve days when the skin had been stored in vaseline or saline gauze The optimum serum concentration for skin storage was found to lie between 10 per cent and 88 per cent, storage in serum alone being definitely harmful. Viability of the stored skin was optimal when the amount of dilute serum used was adjusted so that there was approximately 1 ml for each square cm of skin. Changing the storage medium had a deleterious effect.

The serious shortcoming of all the methods of storage so far described lies in the fact that the viability of the skin declines with storage so that the useful life of grafts so preserved does not extend beyond a month or two at the most. At present this is probably not a very serious clinical disadvantage, though it would certainly become so if means could be devised

to prolong significantly the life of skin homografts. For the experimentalist the shortcoming is more serious

The Storage of Skin in the Frozen State

The first experimental evidence that mammalian skin will withstand freezing to temperatures below the eutectic point of isotonic saline was presented by Mider and Morton (1939) who reported that rat skin which had been *rapidly* frozen to —50°C. (within twenty seconds) and immediately thawed and implanted subcutaneously showed some survival, though better results were obtained by *slow* freezing. They also found that the grafts survived storage at —74°C. for twenty-four hours. Subsequently Briggs and Jund (1944) reported the first successful orthotopic transplantation of frozen skin in mice In their experiments the skin to be frozen was placed in the inner tube of a concentric test-tube assembly with either one or two air gaps The entire assembly was then immersed in pulverized dry-ice at —79°C Successful grafts were obtained after storage at this temperature for one to twenty-four hours They emphasized that *slow* freezing was essential, better results being obtained when two air gaps were used than with one. Their best results were obtained when the temperature of the grafts fell from 0°C. to 15°C within 2 5 to 3 minutes Moreover, they suggested that the more *rapid* the thawing the better, grafts thawed by pouring warm Ringer's solution at 25°C. directly on to them gave better results than grafts thawed by immersing the tube containing them in a water bath at 30°C Few clinical reports have appeared of the use of skin stored in the frozen state Strumia and Hodge (1945) successfully used frozen skin autografts on three patients Split-thickness grafts were placed in a vaccine bottle, just covered with citrated bank plasma and frozen at —20°C. to —25°C, when complete freezing took place within fifteen to thirty minutes, after which they were stored at a temperature below —15°C. The grafts were thawed by immersing the containers in the 37°C. water bath The longest storage period attempted was sixty-

one days It was reported that the percentage of successful takes obtained with the stored grafts was only slightly inferior to that obtained with fresh grafts used as controls. They stated that "instantaneous freezing at very low temperatures is neither necessary nor does it improve the final product."

Billingham and Medawar (1952) have recently carried out experiments designed to determine those conditions of freezing and thawing that result in the minimal possible damage to the structure and viability of thin, split-thickness skin grafts of uniform size removed from the dorsa of rabbits' ears After subjecting the grafts to various treatments *in vitro* their well-being was assessed by transplanting them back in "open style" to an extensive vascular bed prepared on the side of the chest of the animal from which they had been removed initially. Two extreme rates of freezing and two extreme rates of thawing were adopted. *Quick-freezing* was achieved when the grafts, after being flattened on to an extremely thin strip of copper foil by means of vaseline applied to their outer surface, were quickly immersed in an isopentane bath chilled to a temperature below —150°C. With *slow-freezing* the copper strip carrying the grafts was placed in a thick-walled test-tube fitted with an outer one of large bore so that there was an air gap of 3 mm. between them After a preliminary immersion of this assembly in a freezing-bath at —79°C for fifteen minutes, it was transferred to liquid air for a further fifteen minutes, and finally the copper strip was withdrawn and lowered directly into isopentane chilled to below —150°C. as in the quick-freezing procedure *Quick-thawing* entailed plunging the copper carrier bearing the frozen grafts directly into excess Ringer's solution warmed to 37°C, *slow-thawing* was obtained when the metal carrier bearing the grafts was allowed to warm up in an air chamber maintained at 0°C

Before freezing, the grafts were soaked either in Ringer's solution for one hour, or in a 15 per cent solution of glycerol in Ringer's solution which Polge, Smith and Parkes (1949) had found to protect fowl spermatozoa from the otherwise

fatal effects of freezing to low temperatures. It was found that the epithelium of the skin grafts was very tolerant to variation in the rate at which it was frozen, since after either the Ringer or the glycerol pre-treatment it survived either rapid or slow freezing when these were followed by rapid thawing. However, it proved to be much more sensitive to variation in the rate at which it was thawed: rapidly-frozen skin after Ringer pre-treatment did not survive slow-thawing, though pre-treatment with glycerol enabled it to do so Summarized briefly in this manner, the results fail to make it clear that slow freezing was found to be preferable to rapid freezing on the basis of every criterion according to which the well-being of a skin graft may be assessed This conforms with the findings of earlier workers on skin, discussed above, and with those obtained from studies on the freezing of tumour tissues (see Snell and Cloudman, 1943). In the case of skin, the preference for slow freezing is made particularly clear when the fate of epidermal melanocytes (melanoblasts), known to be more sensitive to a variety of physical stresses than Malpighian cells, is taken into consideration. It was found that the melanocytes of rabbit's ear skin pre-treated with Ringer's solution survived neither quick freezing nor slow thawing, slow freezing and rapid thawing being essential. Pre-treatment with glycerol, however, protected these cells against the otherwise fatal consequences of quick freezing but not of slow thawing The protective action of glycerol pre-treatment was made particularly clear by experiments in which epidermal cell suspensions, prepared and grafted according to the methods described in full by Billingham and Reynolds (1952), were frozen. Epidermal cells suspended in Ringer's solution failed to survive even slow freezing followed by rapid thawing, the combination found to be best for intact skin. Nevertheless, after being suspended in Ringer's solution containing 15 per cent glycerol both Malpighian cells and melanocytes survived both quick and slow freezing.

To determine whether glycerol has any demonstrable toxic effects, freshly removed skin grafts were immersed in glycerol

solutions of high concentration with continual stirring Such grafts became optically clear and as stiff as cardboard After rehydration by immersion in Ringer's solution, the grafts were transplanted back to their donors to test their viability. The epidermis of grafts so treated survived exposure to absolute (98 1 per cent) glycerol for eight hours at 0°C. and to 80 per cent glycerol for two hours at room temperature No doubt it would have survived much longer treatments As might be expected, epidermal cell suspensions proved more vulnerable though even these survived exposure to 80 per cent and 98 1 per cent glycerol at room temperatures for as long as thirty-five minutes.

The manner in which glycerol exerts its beneficial effects is unknown. Presumably it achieves some degree of initial dehydration; it lowers the freezing point of water down to a maximum of about —40°C. at a concentration of about 70 per cent, it certainly changes the "grain" of the frozen solid in a conspicuous way, and its viscosity should promote a high degree of supercooling Any of these properties may be important

After determination of the optimum conditions for freezing and thawing skin, the results were applied to the problem of its long-term storage in the frozen state Grafts removed from the dorsal ear skin of rabbits were soaked for one hour in a 15 per cent solution of glycerol in Ringer's solution, after which they were blotted free from excess fluid and placed in small stoppered glass tubes and slowly frozen to and stored at —79°C in contact with solid carbon dioxide contained in a large Dewar flask After storage for various periods up to four hundred days—the longest storage period so far attempted—the grafts were thawed out rapidly by immersion of the tubes in the 37°C water bath while simultaneously pouring in warm Ringer's solution, and the standard transplantation test of survival was carried out In no case was there the slightest perceptible difference between the appearance of the stored grafts and freshly removed grafts transplanted at the same time as controls These results indicate that skin

can be stored in the manner described after pre-treatment with glycerol without apparent deterioration for very long periods. Indeed there seems to be no reason why this storage period should not greatly exceed even the most optimistic estimate of the donor's life. When similar experiments were conducted with skin pre-treated with Ringer's solution the stored grafts on transplantation were invariably found to be inferior to their controls; they regenerated a sparser hair crop showing varying degrees of depigmentation and never recovered the characteristic cosmetic qualities of ear skin

It would be misleading to pretend that the literature on the storage of skin at temperatures below 0°C. is free from inconsistencies. Baxter and Entin (1948), on the basis of clinical transplantation studies, have claimed that skin deteriorates rapidly upon storage below 0°C. and does not survive after exposure to a temperature of —20°C or below for more than two days. Allgower and Blocker (1952) reported failure to obtain epithelial or fibroblastic outgrowth *in vitro* from human skin frozen to —36°C. and stored for fourteen days. Recently, Keeley, Gomez and Brown (1952) reported that dog's skin did not survive rapid freezing—carried out in their experiments by holding tightly stretched skin segments 2–3 cm. above the surface of liquid nitrogen contained in a deep, narrow-mouthed Dewar flask—followed by rapid thawing. However, they claim that when the skin segments were "ultra-rapidly" cooled by plunging them directly into liquid nitrogen, with vigorous agitation to prevent accumulation of gas bubbles, after being first pre-cooled to just above freezing point, a high percentage survival was obtained on subsequent rapid thawing and transplantation. Even better results followed if the grafts were partially dehydrated before ultra-rapid cooling by immersing them in ethylene glycol for twenty seconds to three minutes Grafts so frozen and then stored at —70°C. were reported as showing a high percentage survival after forty-four and after sixty-six days, though not after one hundred and twenty-three days. It is to be regretted that these workers did not include the

effect of slow freezing in their studies and it may be considered questionable whether in fact the rate of cooling in their so-called "ultra-rapid" freezing was faster than the "rapid" freezing as used by Billingham and Medawar (1952). In their investigation, Keeley and his colleagues have followed the teaching of Luyet (1937), who has insistently argued that ultra-rapid cooling leading to vitrification is theoretically to be preferred on the grounds that the formation of ice crystals in the cooling system is thereby prevented, thus avoiding mechanical damage to the cells The advantage of the preliminary dehydration of the grafts is said to derive from the fact that the velocity of cooling required to vitrify aqueous solutions, including colloids, varies with (among other things) the water content. It may be added that Luyet and his colleagues have presented evidence in support of their hypothesis based on the recovery of motility of micro-organisms and of frogs' spermatozoa, and of the irritability of muscle after ultra-rapid freezing and thawing experiments (see Luyet, 1952) Nevertheless, so far as mammalian tissues are concerned, it has been the general experience of those who use continued growth and cell division as a criterion of survival that better preservation is obtained by slow freezing than by fast. This is most fortunate since otherwise the purely technical difficulty of cooling skin sufficiently rapidly to ensure its vitrification before storage would surely preclude its adoption as either a routine clinical or experimental procedure.

The Possibility of Applying Freeze-Drying in the Storage of Skin

There is one case on record in which a "lyophilized" skin autograft is reported as having given satisfactory takes (Webster, 1944) The graft was quickly frozen to −72°C. and then lyophilized; unfortunately no details were given as to the method of dehydration and, more particularly, to what extent the graft had been dehydrated.

A quantitative study of the capacity of thin shavings of

rabbit's ear skin to withstand desiccation from the frozen state has been made by Billingham and Medawar (1952). Since moisture determinations on frozen-dried grafts necessarily involve their complete dehydration, it was obviously impossible to carry out these as well as the skin grafting test of survival on one and the same set of grafts. To solve this difficulty, two similar sets of grafts were removed from adjacent sites on the same animal's ear and dehydrated in separate flasks under similar conditions on the same apparatus Dehydration was carried out for one to twelve hours according to the degree of desiccation it was hoped to achieve One set of grafts was used for the various gravimetric determinations, the other was used for grafting. Both sets of grafts were frozen slowly; the grafts to be used for the viability test were rapidly thawed and rehydrated by pouring into the flask excess Pannet and Compton's solution warmed to body temperature The grafts were either frozen and dried directly, i e. without special treatment, or after pre-treatment with various known concentrations of glycerol in Ringer's solution The untreated grafts were frozen to and dehydrated at —80°C. to —32°C., the glycerol-treated grafts at —22°C. to —25°C , the lower temperature being adopted simply to slow down the rate of dehydration in the absence of glycerol. During the desiccation process the pressure within the apparatus was maintained at 0 005 to 0·001 mm Hg. It was found that the rabbit's ear skin grafts which normally contained about 70 per cent water did not survive a state of dehydration that resulted in a final overall water content of less than about 25 per cent. The pre-treatment of these grafts with glycerol solutions did not increase their capacity to withstand desiccation It may be emphasized that any claim that a tissue withstands desiccation must be substantiated by factual records of its final water content. The outward appearance of frozen-dried skin is most misleading; grafts still containing as much as 25 per cent residual water look, and feel completely dry. It may be added that there is no evidence that any mammalian tissue whatsoever can

withstand drying to below even a 10 per cent residual moisture content

These results suggest that further pursuance of the problem of skin storage in the dry state should be abandoned since adequate methods have been devised for its long term storage in the frozen state

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DISCUSSION

LONGMIRE I should like to ask Dr Billingham about the thickness of the graft that may be preserved by the slow freeze method Is that important in obtaining viable grafts?

BILLINGHAM I see no reason why even full-thickness grafts should not be perfectly viable after preservation by this method Despite the fact that the thickness of a graft will determine the maximum rate at which it can be thawed out—and rapid thawing is essential—even with full-thickness skin a sufficiently fast rate of thawing should be obtainable without difficulty

LONGMIRE· Is the protection afforded by glycerol just a surface

phenomenon, or does the glycerol have to penetrate each cell in the graft?

BILLINGHAM I think the glycerol, to be effective, must diffuse into the graft, and very probably into its component cells. We never treat our grafts for less than an hour with the glycerol solution, which must surely be ample time for it to penetrate them completely. With full-thickness grafts of human skin the period of treatment could certainly be extended without harm.

ALLGÖWER I have been advocating the 10 per cent serum storage of skin, but I would like to say that glycerol storage seems to be much better in long term storage Only there is one point on the clinical usefulness of this storage method very often we want to store the skin for one to three weeks only For this length of time storage in 10 per cent serum is very satisfactory as it can be kept at normal ice-box temperature and because this serum-stored skin can be used immediately on the patients How do you treat the glycerol-stored skin before you put it back on the patient?

BILLINGHAM After rapidly thawing our stored grafts we normally leave them in contact with Ringer's solution for one hour at room temperature before transplantation. This should present no difficulty clinically I should like to emphasize the convenience of this method of storage Although I have had no personal experience of your storage method, I should have thought that in its demands on the surgeon's time and facilities our method was no more exacting. Our refrigerant is solid carbon dioxide (at −79°C) which is cheap and readily available in most countries Our "bank" simply comprises a lagged 5-gallon Dewar flask in which small vessels containing the skin grafts are stored in contact with the dry-ice A 25-pound block of this substance lasts for about three and a half weeks. The skin to be stored is removed aseptically and treated with sterile media, in our experience the addition of antibiotics is wholly unnecessary.

ALLGÖWER Another point: we use tissue culture techniques to assess viability, and are still of the opinion that it is a very exacting test. I wonder how these two kinds of storage would compare as to the tissue culture test. What is the percentage of survival of your grafts when you put them back?

BILLINGHAM I am unable to answer in terms of percentage survival. We have studied detectable differences between the appearance of our stored skin grafts after transplantation and that of freshly cut skin grafts transplanted on the same occasion as controls After storage following glycerol pre-treatment, by the methods I've described, in no case have we found the stored grafts to be inferior to their controls after transplantation

POMERAT. I think the use of the tissue culture method as a *final* test, especially for exploratory operations, is very important, because so often one can be fooled by these *in vivo* methods. Very often an adjacent tissue may come into the picture and confuse the issue For example, in tissue cultures of dermis, a cut sweat gland duct may proliferate and pour out a veritable island of epithelium that looks identical to that

which comes from the middle of the epidermis I think sometimes in the denuded skin we have repair arising from the ducts of glands which are of course of the same point of origin from stratum germinativum But when you have true emigration *in vitro*, you can then be absolutely certain that the material is viable and is not a derivative of some adjacent area

MEDAWAR But mere cellular survival is not the only important criterion of the persistence of the graft after storage in a state as good as new One has to consider such changes as take place in the fibrous endoskeleton, which will determine the pattern and density of hairs, for example. The criterion that Billingham and I have been using is quite simply the restitution of skin to a state as good as new in every respect, and I don't see how tissue culture can improve on that

POMERAT No, I agree with you—unless you had a formation *de novo*, for example from a gland base of an island, but I think it would take a good deal of time to produce that

MEDAWAR In theory that is a danger, but our survival test in fact precludes that These grafts are put right into the middle of a great big raw area, stripped right down to the panniculus carnosus muscle. Now I don't believe in metaplasia of, shall we say, mesenchymal cells in the epidermal cells

BILLINGHAM I might add, Prof Pomerat, that in taking the rabbit we were fortunate, because sweat glands do not occur.

ROGERS From a historical standpoint I think it is interesting that an American surgeon, Z J Lusk in 1895, used corrosives to raise blisters on the epithelium of some of his patients and then, excising the bilateral material, he dried it and stored it in a bottle He carried the bottle containing dried blisters on his rounds, applying them indiscriminately on slow-healing or second and third-degree burn defects Now these dried blisters or blistered epithelium were supposed to have actually grown, according to Dr Lusk, as viable epithelium once they were transplanted Although his follow-up studies are poor in the three articles which he wrote, from a standpoint of tissue we might consider the possibility that Dr Lusk's dried, blister epithelium may be somewhat similar to the freeze-dried type of grafts We don't expect viability or as you have implied, surviving cells in a freeze-dried graft and yet the U S Navy hopes to make clinical use of these freeze-dried grafts in any number of surgical problems

PATE We have dehydrated our freeze-dried skin to a total water content of less than 1 per cent, and are morally certain that these pieces are completely dead However, after putting them on about 20 patients, we have not found any surgeon yet who can tell which is the freeze-dried skin and which is the fresh homograft. We know it's dead but it fulfils its mechanical function

BILLINGHAM With regard to the use of these frozen-dried skin grafts, I should have thought that their surfaces would have been moist soon after transplantation since the epithelium presumably disintegrates fairly rapidly as a consequence of autolysis and other processes

PATE In fact, the opposite is true, it's very dry

been inserted at about 2 mm. a day—only a little slower than the 3 5 mm. a day at which they advance down the distal stump of a nerve which has been severed and reunited immediately (Sanders and Young, 1942; Sanders, 1942; Gutmann and Sanders, 1942). Recovery of reflex function and cutaneous sensibility also take place at a time and with a final quality comparable to that obtained following severance and immediate reunion.

Histological examination of autografts shows that re-innervation proceeds very much as in an ordinary distal stump, although proliferation of Schwann nuclei and break-up of the myelin takes place more slowly than normally. In the terminal stages of regeneration, however, an autograft resembles a normal distal stump very closely indeed.

Homografts

Pieces of fresh nerve transplanted to fill a gap in a nerve of another individual of the same species become, like autografts, firmly united with the host nerve, although thereafter their behaviour is very different. In favourable circumstances the distances reached after fifteen or twenty-five days by nerve fibres growing through such grafts are as great as those through autografts. Most grafts, however, show far shorter distances of outgrowth, and when treated statistically the data show evidence of a significant variability not under experimental control (Sanders and Young, 1942). Recovery, both of motor function and of cutaneous sensibility, can nevertheless follow the use of short homografts in the rabbit, e g six out of seven such grafts investigated by Gutmann and Sanders (1942) recovered motor power of a quality little inferior to that given by autografts Four of the six animals which showed motor recovery also had some return of cutaneous sensibility.

The variability in the distances reached at a given time by new fibres growing through homografts is reflected in their histological appearance Wallerian degeneration takes place more slowly in homo- than in autografts so that unbroken myelin segments can be found as late as twenty-five days

(Fig 1). The result of this delayed breakdown of the contents of the graft can be seen at sixty days when a graft may contain large spaces full of active macrophages (Fig. 2), even two hundred days after grafting similar, though smaller, macrophage-filled spaces remain (Fig. 3). It is uncertain, however, how far these later appearances reflect the process of Wallerian degeneration (which although slow is fairly normal in its early stages) and how far they result from a deep seated reaction from the host to the graft which causes the latter to be destroyed and replaced partially by the host tissue (see below, pp 181-182).

By far the most striking feature of homograft histology is their invasion by lymphoid cells The degree of invasion is very variable. In the best case infiltration is only minimal but in less favourable instances the whole graft becomes distended by a mass of lymphocytes and its internal architecture considerably disturbed (Fig. 4); eventually there are patches of necrosis, in extent proportional to the intensity of this reaction. Although the lymphocytic infiltration subsides fairly quickly there is inevitably a certain amount of fibrosis and a less uniform innervation than in autografts. Although new nerve fibres are able to penetrate homografts and to become myelinated within them (Fig. 5) parts of such grafts, usually at the centre, contain either no, or only small, nerve fibres (Gutmann and Sanders, 1943) However, in spite of such unfavourable features nerve homografts in the rabbit are still able to conduct nerve fibres across a two centimetre gap in sufficient numbers to produce a return of motor function and of sensibility only a little inferior to that of autografts.

The nature of the homograft reaction

The apparent success of nerve homografts in animal experiments is not paralleled by any similar result in man (Sanders, 1942, Seddon and Holmes, 1944). Very few human nerve homografts have been followed by any recovery of function which could be unequivocally ascribed to the affected nerve, and sections of such grafts, removed at long intervals

after insertion, have shown them mainly converted into f
strands. The reason for the discrepancy between the r
of homografts in man and in experimental animals c
found in the nature of the host reaction to homografts

In the case of skin transplants Medawar (1944, 194
shown that homografts behave at first very like autog
the grafts healing securely, and even giving rise to epi
proliferation. However, after a variable period, an
inflammatory reaction sets in, and the whole nati
population of the graft is destroyed. The inflammator
cess includes first of all vascular and lymphatic prolifer
a massive invasion of the graft by lymphocytes and r
cytes of host origin, severe œdema, and a general mobiliz
of mesenchyme cells At the close of this phase, a stagn
and obliteration of the vascular system of the graft, tog
with the death and necrosis of its constituent cell popul
take place. Later, homografts are invaded anew by
vessels from the host, and lymphocytes and monocytes
through their walls to establish a secondary cell popul
within the graft

The whole reaction of the host to the grafted foreign t
is of the type of an actively acquired immune reaction,
consequently, depends quantitatively upon the amour
foreign tissue transplanted Thus, a single small homog
of skin may survive many days without destruction, v
larger amounts of tissue—whether transplanted as a si
large, or many separate small grafts—undergo a much r
rapid breakdown Moreover, "second-set" grafts from a g
donor to a host already immunized by grafts from that do
break down even more quickly Such observations ar
particular interest since, in the case of nerve grafts, one of
main features distinguishing human and animal homog
experiments has been the size of the transplants used. M
animal experimenters have used small thin grafts 2–3
long, while most of the grafts used to repair human ne
defects have been at least 8–10 cm long, and of correspo

"dosage" of foreign tissue than has been used in animal experiments, and, provided nerve homografts behave similarly to skin, the more rapid breakdown of such large grafts might be expected adversely to affect recovery It is interesting to note in this connection that the only human nerve homografts to show any convincing evidence of recovery were three of Duel's (1933) six cases of facial nerve homografting In all of these the dosage of tissue was low; the nerve transplanted was of small diameter, and the gap to be bridged was short.

A dosage effect may also be inferred from the data of fourteen human nerve homografts removed and examined histologically after failure to regenerate (Seddon and Holmes, 1944, Barnes, Bacsich, and Wyburn, 1945, Spurling, Lyons, Whitcomb and Woodhall, 1945) In Fig 6 (p 180) the distances reached by nerve fibres growing through such grafts is plotted against the length of the graft used and it will be seen that there is, generally speaking, an inverse relationship between the size of the graft and the distance reached by nerve fibres growing within it The relationship may in fact be closer than that shown in the diagram The cases of Spurling *et al* (1945), which together form the group of short grafts in which some outgrowth had occurred, were most of them excised after shorter periods than those of the other authors Had they been allowed to remain longer in the host they might have shown still greater distances of outgrowth. The 18-cm graft of Barnes *et al* (1945) is exceptional in that it was penetrated by new fibres for a distance of 25 mm , while neither of the large grafts of Seddon and Holmes (1944) showed any regeneration whatsoever However, the whole reaction to this graft was much milder than in any of the other grafts studied and may indicate a closer compatibility between the tissues of donor and host than in the other cases

The dosage effect has also been studied in the rabbit by comparisons between long (5-cm.) and short (2-cm) homografts in the peroneal nerve, the fate of the grafts being followed for as long as four hundred days after operation (Sanders, 1953) The distance reached after thirty days by

new fibres growing through 5-cm homografts was less than that reached five days earlier when 2-cm. grafts were used. Indeed, in three out of fourteen long grafts, fibres had not crossed the upper junction at this time.

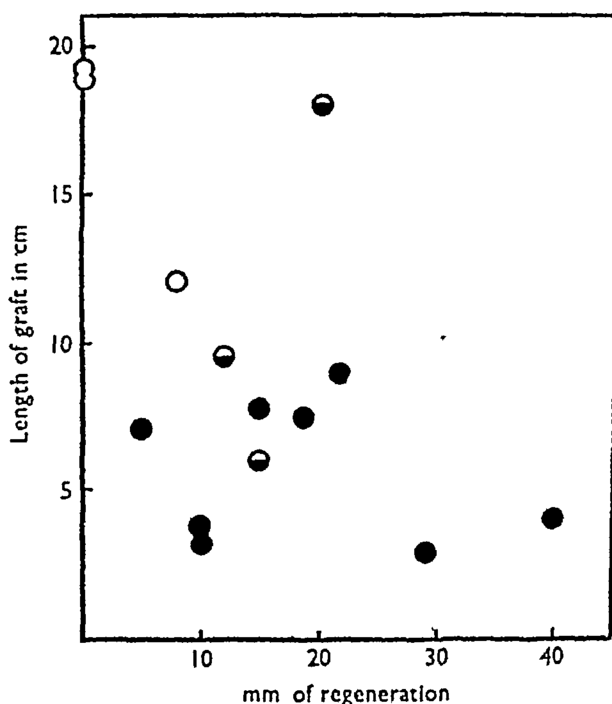


FIG 6 Distances reached by nerve fibres growing for long periods through homografts of various lengths in man. (Filled circles cases of Spurling *et al* (1945) Half-filled circles cases of Barnes *et al* (1945) Empty circles cases of Seddon and Holmes (1944))

The return of motor function was delayed in onset (eighty-five to two hundred and fifty-five days) in long as compared with short grafts (seventy to one hundred and twenty-five days), moreover, the quality of motor recovery finally attained was much inferior. No animal bearing a long graft showed any return of cutaneous sensibility whatsoever

Histological investigation of the inflammatory changes



FIG 1 Abnormal myelin degeneration in a rabbit nerve homograft twenty-five days after insertion (Weigert stain)

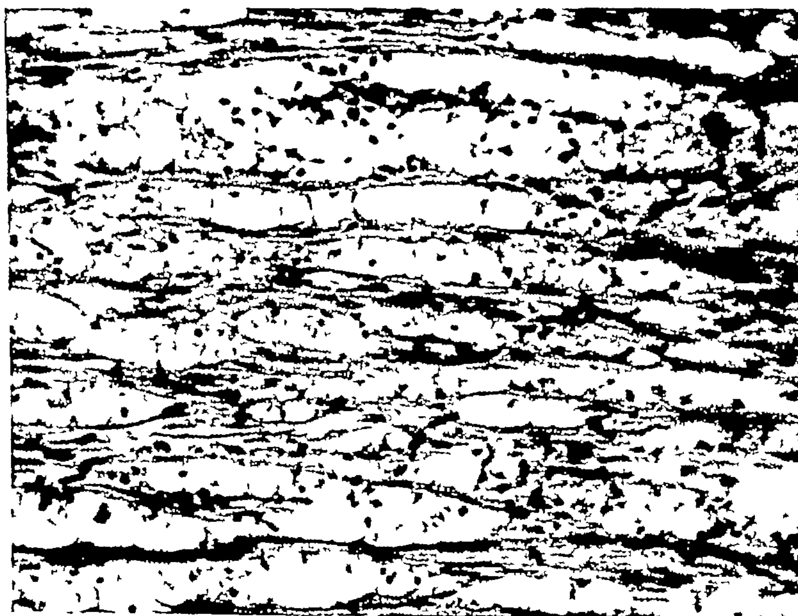


FIG 2 Abnormally large macrophage-filled spaces in a homograft sixty days after insertion (Bodian-Masson stain)

[*To face page* 180



FIG. 3 Residual macrophages in a homograft two hundred days after insertion (Masson stain.)



FIG. 4 Severe lymphocytic reaction in a 2 cm. homograft twenty-five days after insertion (Bodian Masson stain.)



FIG 5 New myelinated nerve fibres in a homograft twenty-five days after insertion (Weigert stain)

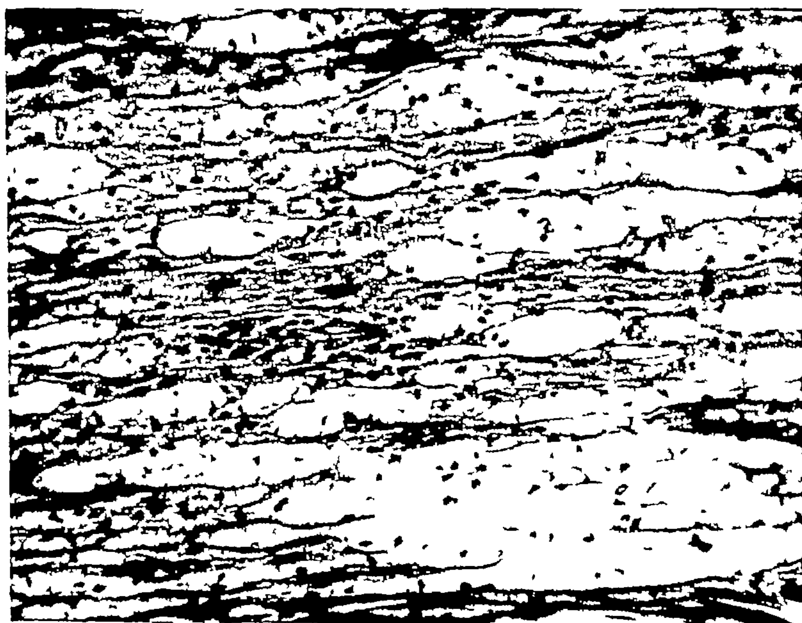


FIG 7 New nerve fibres and Schwann cells in the upper part of a nerve homograft twenty-five days after grafting (Bodian-Masson stain)

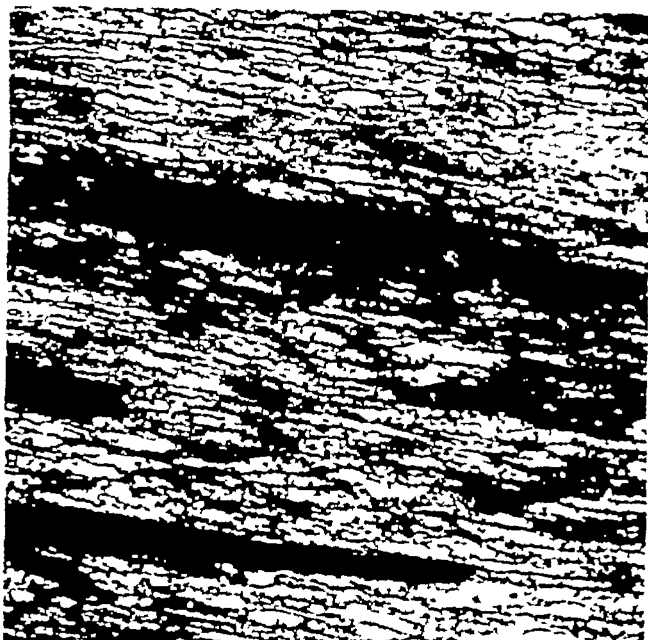


FIG 8 Stagnant, dilated, blood vessels in the upper part of a 5 cm. homograft thirty days after grafting (Mallory stain)



FIG. 9 Hemorrhage within a homograft inserted thirty days previously (Mallory stain)

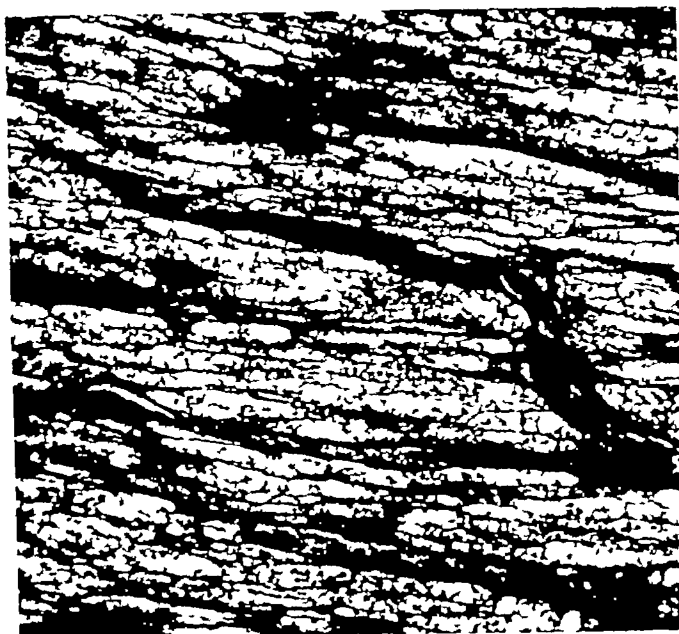


Fig 10 Necrosis of the native cell population of a homograft thirty days after grafting, note also dilated, stagnant blood vessels (Mallory stain)



Fig 11 Schwann cell strands of host origin in the lower part of a 5 cm homograft thirty days after grafting (Masson stain)

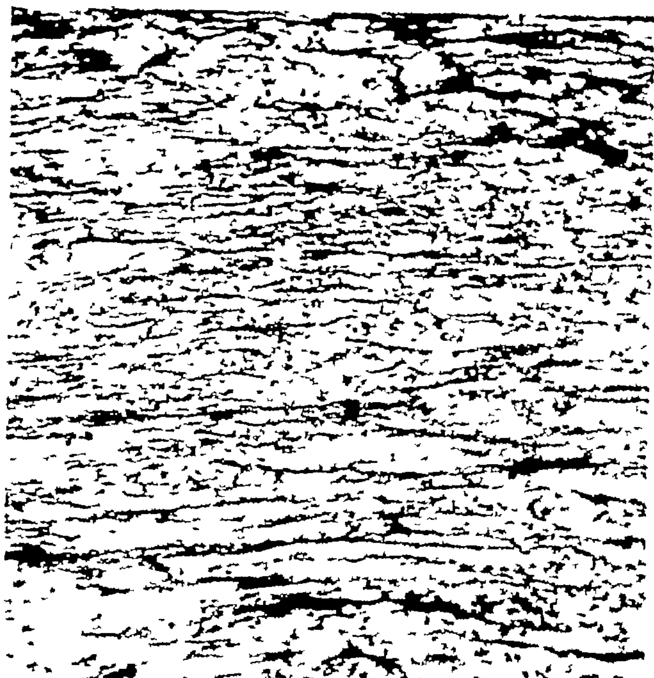


FIG. 12 Necrotic segment from the middle of an 8.5 cm. homograft thirty days after grafting (Masson stain.)

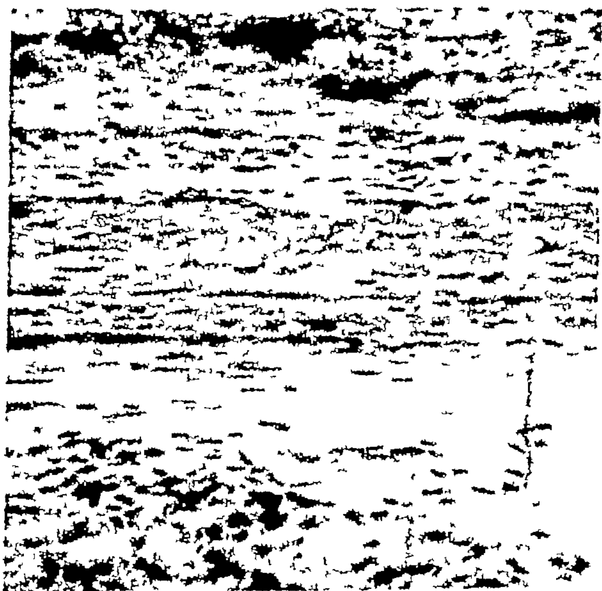


FIG. 13 Fibrosed central part of a 10.5 cm. homograft [illegible] days after grafting (Masson stain.)

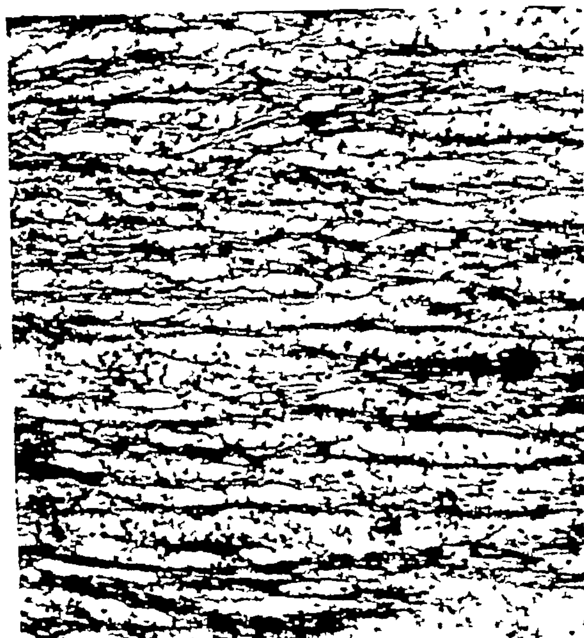


FIG 14 Excessive macrophage population in a homograft stored for three weeks at 0°C in Ringer's solution before grafting (Bodian-Masson stain)

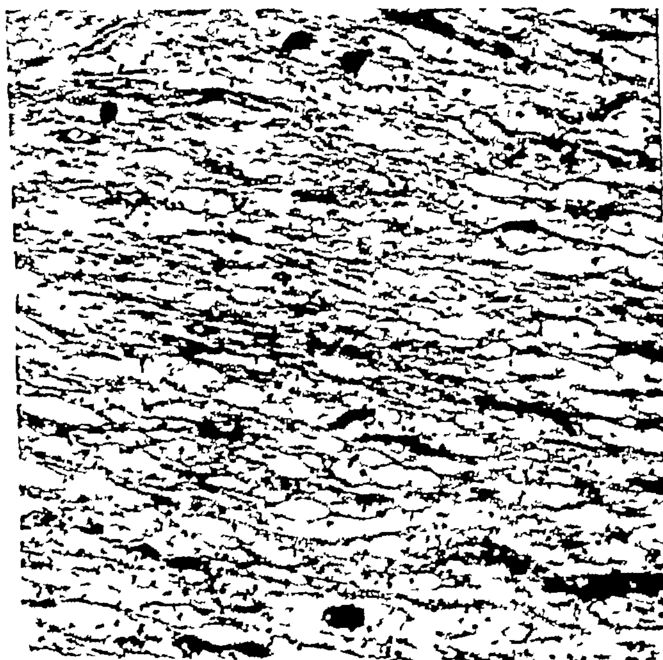


FIG 15 Frozen-thawed homograft twenty-five days after grafting (Bodian-Masson stain)

which take place within nerve homografts has shown that the response of the host tissue to such grafts resembles that seen in skin homografts (Medawar, 1944, 1945), although with nerves the reaction is complicated by the activity of the nerve fibres and Schwann cells of the host nerve into which the graft is inserted. In the case of a nerve homograft, primary union between the graft and the host nerve takes place Wallerian degeneration begins within the graft, together with proliferation of its Schwann cells New blood vessels, coming mainly from the ends, grow into the graft, and nerve fibres from the central stump cross the upper junction, start to travel down the Schwann tubes of the graft (Fig. 7) and may begin to myelinate within it (Fig. 5) The lower junction is made by Schwann cells from both the graft and the peripheral stump.

Parallel to these changes, and at a rate and with an intensity dependent upon graft dosage, the inflammatory reaction builds up. Mononuclear cells pour out from the blood vessels into the graft, which becomes œdematous. The reaction is most intense at the junctions, which at twenty-five days are markedly swollen and packed with lymphoid cells, large, dilated blood vessels can be found within the graft, especially in the neighbourhood of the junctions (Figs 8, 10). At the height of the reaction, the walls of the vessels break down, and local hæmorrhages occur within the graft (Fig. 9) At this time the native cell population of the graft is destroyed (Fig. 10) At this time, therefore, new nerve fibres and Schwann cell strands from the host nerve, which have invaded the graft from its two ends, are exposed to an environment in which the only structures maintaining the continuity of the nerve are the collagenous tubes of the graft. In short grafts this is without serious effect, since fibre and Schwann cell continuity is established throughout the length of the graft by cells of host origin in the period before breakdown takes place (Fig 11). These structures seem relatively unaffected by the short period of ischæmia which results from the breakdown of the blood vessels of the graft, since short nerve

homografts removed at long intervals after insertion closely resemble autografts, having been repopulated by cells from the host nerve. The accumulations of macrophages seen in such grafts in the later stages probably have to do with the removal of the dead and necrotic remnants of the original graft contents. The fate of the graft collagen is less certain. Medawar (1945) found the collagen of skin homografts to be removed eventually and substituted by host collagen, although this took place much later than the destruction of the cells of the graft; phagocytosis of the graft collagen fibres did not take place. The collagenous framework of nerve grafts may also be replaced by host collagen. If so, the new collagen must be laid down in the pattern of the old, since the latter undoubtedly acts as a guide for the nerve fibres and Schwann cells as they traverse the graft. This might account for the slight interstitial fibrosis seen in the late stages of successful low dosage homografts.

In long grafts the bigger dosage of homologous tissue causes the inflammatory reaction to come on earlier and to be more intense Primary vascular breakdown, and destruction of the native cell population of the graft takes place before it has been penetrated throughout by blood vessels, Schwann cells, or nerve fibres Regions thus exist in the middle of a long nerve homograft which have been without blood supply ever since transplantation (Fig 12) Such a region later becomes heavily collagenized and can act as a barrier to regeneration (Fig 13). As a result, a long nerve graft examined at a late stage often consists of a thick fibrous strand, at either end of which are segments resembling normal nerve. that at the upper end looks like a re-innervated distal stump; that at the distal end resembles a degenerated distal stump of long standing (Holmes and Young, 1942). Similar histological appearances were found in human nerve homografts removed following the failure of regeneration What counts most against the success of a large nerve homograft is the fibrosis of part or all of the graft The feature of the homograft reaction which seems most damaging from this point of view

is the breakdown of the blood vessels growing within the graft and not the death of the constituent cell population It is thus possible that some treatment of homografts which would modify the "homograft reaction" in the direction of avoiding primary vascular breakdown might improve the prospects for homografting whether or not it destroys the cells of the graft

The results of attempts to modify the homograft reaction

Very little work has been done in the past with this specific aim. Most authors who have investigated procedures for the storage of nerve grafts have been concerned with providing "banks" of stored nerves for the treatment of war casualties Assessment of the results of storage procedures has of necessity been pragmatic Certain storage procedures however are of some interest since they do seem to modify the extent of homograft reaction Sanders and Young (1942) and Gutmann and Sanders (1942) studied the behaviour of grafts of rabbit nerve which had been simply kept in Ringer's solution at 0°C. for one or two weeks before transplantation. Nerve fibres grew readily through such grafts, occasionally as fast as through autografts. Motor and also sensory recovery followed, with a quality definitely superior to that of fresh homografts of comparable size, but inferior to that of autografts. A striking effect of storage in Ringer's solution was a reduction of the amount of lymphocytic invasion—the extent of the reaction became progressively less with an increasing period of storage—with a result that grafts stored for two or three weeks made very clear junction with the host nerve and were accompanied by the minimum of nerve fibre deviation. However in the early stages of re-innervation of such grafts there was an excessive penetration by macrophages; this being most marked in the grafts stored for longer periods (Fig. 14) However, at late stages there was no sign of this macrophage reaction and the grafts resembled normal distal stumps containing many Schwann cells, and new fibres in process of myelination The Schwann cells were undoubtedly

of host nerve origin. As storage is prolonged it probably results in the death of more and more of the cells of the graft, the pronounced macrophage invasion seen in grafts stored for longer periods being concerned in the removal of this dead tissue.

Weiss (1944) and Weiss and Taylor (1945) found a similar reduction in the reaction to homografts which had been dried from the frozen state and subsequently rehydrated. Not only did new nerve fibres and Schwann cells grow through such grafts but functional recovery in both cats and monkeys was found following their use However in all their experiments short grafts (less than 3 cm) were used and experiments with longer grafts have failed to show any superiority of frozen-dried over fresh homografts (Sanders, 1954). We have compared the functional results of 5-cm. frozen-dried homografts of rabbit nerve with those of comparable fresh nerves. There was no significant difference either in the time of onset or the quality of the functional recovery achieved Histologically there was little to choose between fresh and frozen-dried homografts when examined in the late stages of regeneration. The latter, although penetrated by a fair number of nerve fibres, were as heavily collagenized as fresh grafts. In the early stages of regeneration there was no great lymphocytic invasion as seen with fresh grafts. It thus appears probable that the freeze-drying exercises its effect in the same way as simple storage in Ringer's solution, i e by killing the native cells of the graft, and this accounts for the absence of lymphocytic invasion in the early stages Alteration of the chemical nature of the graft as a result of the drying procedure, however, may account for the lack of success of such grafts. Sanders (1953) investigated the behaviour of grafts in which the cell population had been deliberately reduced by five successive cycles of freezing (−70°C) and thawing (37°C) before transplantation This procedure was chosen as a way of killing cells with the least effect on the proteins of the graft Table I shows the distances reached after thirty days by fibres growing through 6 5- to 8 5-cm grafts in the

case of fresh and frozen thawed homografts. The distance reached by fibres growing through fresh grafts was highly variable, the mean distance being 9 4 mm Fibres had grown somewhat further through the frozen thawed grafts (mean

TABLE I

COMPARISON OF DISTANCES REACHED IN THIRTY DAYS BY NERVE FIBRES GROWING THROUGH EITHER FRESH, OR FROZEN/THAWED, NERVE HOMOGRAFTS

Length of Graft	*Distance of outgrowth in mm*	
	Fresh Grafts	*Frozen/thawed Grafts*
8 5 cm	0	12
	8	15
	8	17
	15	18
	16	18
	18	19
	37	21
6 5 cm	0	11
	0	15
	2	17
	4	19
	4	20
	10	20
	10	58

Mean distance of outgrowth (fresh grafts) = 9 4 mm
Mean distance of outgrowth (frozen/thawed grafts) = 20 mm

Analysis of variance of above data —

Item	*Degrees of Freedom*	*Mean square*	*t*	*P*
Fresh v frozen-thawed grafts	1	783	6 692	0 001
8 5 m v 6 5 cm grafts	1	87		
Residual variance	25	117		

distance of outgrowth=20 mm.) and the difference between the two types of graft was statistically significant. Histologically the frozen thawed grafts did not elicit a homograft reaction although they were penetrated by Schwann cells and new fibres (Fig 15). There was however an excessive

number of macrophages in these grafts, concerned presumably with the removal of the dead graft contents. Functional results of this latter procedure are not at present available and further work will be necessary before the clinical prospects of such frozen thawed grafts can be determined.

All the histological appearances seen in nerve homografts are consistent with the following view —

1 The constituent cells of a homograft are destroyed as a result of an immune reaction on the part of the host, the severity and the rapidity of whose onset depends both upon the amount of tissue transplanted and the genetic relationship between donor and host

2. At the height of this reaction the new blood vessels which have grown into the graft from the host nerve break down

This leads to a variable degree of avascularity depending on the size of the graft and consequent fibrosis. Until there have been further investigations of the details of this reaction and treatments have been devised for its modification, the use of any kind of nerve homograft in man is to be discouraged.

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DISCUSSION

EICHWALD By what method did you determine the rate of travel of the regenerating fibres?

SANDERS An extremely simple and crude one The animal was anæsthetized and the nerve carrying the graft severed peripherally and freed from its bed It was then pinched with fine, smooth-tipped forceps at 1 mm intervals from the distal end upwards, until the animal first gave a sensory response The distance of this point from the upper graft junction was then recorded and the piece of nerve including the first positive pinch removed and checked histologically for the presence of nerve fibres This method in fact measures the distance reached by the fastest growing sensory axons (Gutmann, Gutmann, Medawar and Young, 1942) *

EICHWALD You never re-pinched the same graft, you just used it once?

SANDERS We checked that, yes We went beyond the necessary sensory response

HYATT Isn't it possible that the method might have encouraged intraneural fibrosis?

SANDERS Pinching the nerve was only done as part of a terminal experiment Immediately after the distance of fibre outgrowth had been so measured, the whole graft was removed for histological study No pinched nerve was allowed to remain in the animal long enough for secondary histological changes to occur As regards pinching itself, the genuineness of the first positive response was always checked by one or two more pinches more proximally

HYATT Did Weiss indicate or intimate that the frozen-dried nerves lived? What was his freeze-drying procedure?

SANDERS My impression is that he makes no definite statement concerning survival of the grafted elements The technique used involved quick-freezing in isopentane chilled with liquid nitrogen, followed by low-temperature vacuum drying over phosphorus pentoxide Our procedure was the same

ROGERS I am rather disturbed about the dosage phenomenon I certainly believe it can be demonstrated in animals but I'm not yet convinced that it applies in humans, at least as far as skin homografting is concerned Clinically I have seen split-thickness skin homografts about two inches wide and two inches long applied, which have sloughed in three weeks even though the dose was small and the genetic relationship was close (mother donated skin to cover daughter's burns) Another mother donated for her daughter roughly 25-30 square inches of split-thickness homografts, which survived for two and half months I know that other variables might be considered, e g the closeness of a hæmatological relationship and so on, but I wonder whether the dosage phenomenon can be taken as seriously in clinical practice as it can in animal experimental work?

*J exp Biol , 19, 14

SANDERS I can't speak on the subject of skin, and for nerve, of course, the data on dosage are extremely scanty Clinically, a nerve graft is not often used unless the nerve gap to be closed is enormous, which automatically implies a large dose of homologous tissue Only three human nerve homografts (Duel, 1938) have shown unequivocal evidence of recovery of function, and these were comparatively short grafts in the facial nerve in the temporal canal By contrast, almost all the grafts used in animal experiments have been short and represent a much smaller dosage of homologous tissue This fact alone is adequate to explain the discrepancy between clinical experience and the results of animal experiment as far as nerve homografts are concerned Dr. Longmire, do you remember how long were the homografts used by Davis in his animal experiments? They were short grafts, weren't they?

LONGMIRE I think they were 2½ cm in length.

MEDAWAR: Dr. Sanders' experiments have a very considerable cautionary value—they illustrate one of the occasions on which animal experiments can be totally misleading, when applied to human beings As I see it, the essentials of his argument are first of all that the rate of advance of nerve fibres is just about the same after injury in man and in rabbits, and, second, that the tempo of immunological response is also about the same And therefore one can't compare gaps of proportionate size in man and in rabbits, it is the *absolute* size of the gap that matters. This is therefore a case in which one can't use the rabbit as a proportional model for studying reparative processes in man

LONGMIRE In the immune response which these nerves provoke, have you any impression, Dr Sanders, of the importance of the Schwann cells or of the nerve fibres themselves, the contents inside the sheath?

SANDERS I'm afraid I have no direct evidence at all concerning that point We assume that an immune response is occurring during nerve homografting by analogy with Prof Medawar's work with skin, and on the fact that we were also able to demonstrate a second-set phenomenon in the case of nerve grafts. In nerve homografts the axons and myelin break down as they do in all nerve fibres separated from their cells of origin, the Schwann cells appear to be destroyed during the homograft reaction, the collagenous tubes, as in the case of skin, appear to last longer While the reaction in its violent phase appears to affect principally the Schwann cells, I do not know how many of the elements are concerned in stimulating the immune response of the host

LONGMIRE I was wondering about the possibility of allowing the material inside the sheath to be removed in the host before making the transplant.

SANDERS My main point is that, where nerve is concerned, the important phase of the homograft reaction is not necessarily the destruction of the native cell population, since new Schwann cells can come into the graft from the host What *does* matter is the breakdown of blood vessels A race seems to take place between blood vessels growing into the graft from both ends and the build-up of the homograft reaction In short grafts the vessels get right through in the first

wave, so that you get a graft which is at any rate intermittently vascular, in which the host nerve fibres and Schwann cells are able to survive. In a long graft the blood vessels never get to the middle before breakdown occurs, so that you have a central segment which just goes into a mush and eventually becomes fibrosed

THE PRESERVATION OF ARTERIES AND OTHER TISSUES FOR CLINICAL USE

CHARLES ROB and H H. G. EASTCOTT

We intend in this paper to concentrate our remarks upon man and will only touch in brief fashion upon our work in experimental animals Whilst most of the time at our disposal will be taken up with a discussion of the preservation of arteries, we wish to put forward some of our ideas and data on the preservation of human endrocrine glands, the human cornea and the rat's and rabbit's trachea

Arteries

At St. Mary's Hospital we have had a human frozen artery bank in operation since the summer of 1951 and the results have been satisfactory. The technique of artery banking which we use is based on principles used in the food preservation trade and is patterned on the original work of Charles Hufnagel. The grafts are taken from donors dead for less than twelve hours, and young enough to have macroscopically normal arteries, and the body must have been placed in a cold chamber within two hours of death. A full aseptic ritual is used as for a surgical operation Suitable segments of arteries are excised and placed in a bowl of sterile isotonic saline. The grafts are then placed in sterile pyrex tubes and frozen in a mixture of alcohol and dry ice at a temperature of -70°C. for five minutes. After this they are stored in a deep-freeze at a temperature of -70°C. or less This temperature is achieved by incorporating dry ice in a special cork lagged box within the deep-freeze. When required for use the grafts are removed and thawed rapidly by pouring sterile isotonic saline at 39°C. into the tube until the graft floats free when it is tipped into a bowl of saline at this temperature

After such treatment these grafts are indistinguishable microscopically from normal arteries, but they are dead. That they may survive the original quick freezing process was shown by Hufnagel and Eastcott (1952) in two ways: firstly, a series of tissue culture experiments showed a moderate growth of fibroblasts in seven out of 20 cultures of vessel wall frozen in liquid nitrogen and thawed immediately; and secondly, applying Medawar's criterion of tissue survival (reimplantation into the donor) whole segments of carotid artery were frozen and thawed in the way described and when examined three months after autografting showed normal function and histology. But 18 cultures from arteries which had been banked as described for periods of between five and one hundred and sixty-six days showed no growth.

The ideal artery or tissue bank should fulfil the following conditions.—1. Perfect safety and sterility. 2. Prolonged or indefinite storage. 3 Easy maintenance. 4 The grafts should be transportable from hospital to hospital and town to town. 5 In the case of certain tissues the graft should be alive when placed in the recipient

The artery bank at St Mary's Hospital, which is the first human frozen artery bank to be established in the world, gives safety and sterility Prolonged storage in human work is of great importance because of the difficulty of getting donors and with the earlier artery banks this proved to be a source of difficulty. Peirce found that in his bank more than 90 per cent of the grafts were wasted, but with a frozen artery bank the wastage should be minimal, and we have a utilization rate of at least 95 per cent. Easy maintenance is a feature of storage of frozen tissues, all we do is to replenish the dry ice every third day Transport from hospital to hospital is useful, we have sent grafts from St Mary's Hospital to Colchester, a distance of 60 miles. An advantage of this method is that unused grafts can be returned to the bank and are not wasted Our bank does not provide live arterial grafts but since seeing the publications of Dr. Parkes and his colleagues

we have attempted to remedy this defect for glandular and other tissues.

In arterial grafting the results do not appear to be influenced by the viability of the graft. Homografts nearly always die in the host and arteries are no exception Artery grafts, whether alive or dead when placed in the host, merely act as a scaffold into which the host tissues can grow. In the case of arteries it is possible that the new intima is deposited as cells from the blood stream, although growth also occurs from the ends of the host vessel, the media and adventitia are replaced by fibrous tissue and the elastic lamina appears unchanged when examined through a microscope, whether it is alive or not, we do not know. The results of using frozen arterial homografts of this kind in the femoral and popliteal arteries of man are shown in Table I. Other methods of reconstruction such as autogenous vein grafts have not been mentioned, but in our opinion they have many advantages over artery grafts for the peripheral vessels.

Endocrine Tissues

We now store endocrine tissues in glycerol saline, as recommended by Polge, Smith and Parkes (1949) In this way cells survival may possibly occur and the uses of a frozen tissue bank can be extended beyond the conventional fields of artery, bone, corneal and cartilage grafting.

The Adrenal

The development of surgery in this field to include the operations of total and subtotal adrenalectomy for such conditions as Cushing's syndrome, advanced carcinoma of the prostate, and hypertension, has opened up an interesting field for the clinical use of the storage of glandular tissues. When we do a total or subtotal adrenalectomy today we preserve slices of the gland in various ways, including glycerol saline, they are carefully labelled so that should the need arise we can return the patient's own gland as an autograft, placing thin slices in the muscles under local anæsthesia. We have

several such glands in our bank, but so far none of the patients has required an autograft. After six months' storage of such glands the histological appearance is still normal but cell survival has not been proved after this length of time.

The experimental side of this subject suffers from the difficulty of getting a suitable animal preparation for an adequate therapeutic trial of an autogenous adrenal graft Our efforts with the rat have so far been largely unsuccessful, but after hearing Mr Jones's communication we are encouraged to try again

Trachea

Tracheal homografts have been inserted in 18 rats and three rabbits Eight of these rats received a fresh homograft of a

Table I

Frozen arterial homografts used		18
Claudication	11	
Gangrene	2	
Immediate failures		4
Return of distal pulses		9
Subsequent relapse		3
Condition maintained during 2–18 months		6

complete segment of trachea 4 rings in length Five of these eight rats had to be sacrificed between five and ten days after the operation because the graft became necrotic and obstructed the airway, two rats lived for one hundred and thirty days and one hundred and four days respectively before they developed strictures of the trachea, and one was killed on the one hundred and seventy-fifth post-operative day with a satisfactory airway. Therefore only one fresh homograft in eight worked entirely satisfactorily.

Segments of trachea were preserved by freezing in the same way as we preserved arteries Ten rats and three rabbits received such a frozen and preserved tracheal homograft All the grafts became necrotic and every animal had to be sacrificed before the twenty-first post-operative day

The fresh tracheal homograft usually failed, the cartilage rings sometimes survived and provided a framework for a new tracheal wall The frozen trachea became necrotic on every occasion, but perhaps after preservation in glycerol saline an occasional successful result might be obtained.

Cornea

Arrangements for supplying human corneas, although much easier since the Corneal Graft Bill, 1952, must still be made on a day to day basis; the storage life of corneal grafts at refrigerator temperatures is limited to a few days. Grafts have been used with success after four weeks but this is exceptional. Little has been published on their preservation by freezing Katzin (1947) found that nine frozen-dried corneal grafts, though clear when rehydrated, all became opaque within twenty-one days of insertion into the patient He cited the Russian literature on survival of corneal cells after freezing, as judged by tissue culture. At St Mary's Hospital we are now using the glycerol saline medium for corneal grafts The grafts are placed in the glycerol solution for one hour and then frozen at —79°C. for five minutes, after which they are stored in the artery bank until needed They are thawed by placing the container in a thermos jar of water at 40°C. Preliminary results in lamellar grafts are encouraging the ophthalmic surgeons find them satisfactory to cut and to suture; and so far six have remained clear for periods of up to four weeks. Tissue culture studies are being carried out on these grafts by Mr. S. E. Smith of Dr Parkes's department at the National Institute for Medical Research, Mill Hill It is impossible at this early stage to say whether results will equal those of fresh grafts, but if the cornea can be stored at —79°C. in glycerol-saline for a prolonged period we may expect to see a great increase in the scope of this part of ophthalmic surgery

Conclusion

A frozen artery bank works well. The discovery of Parkes and his colleagues that frozen storage in glycerol saline

permits cell survival opens up fields of great interest to the surgeon Delayed autografts may become possible and elective homografts simplified. The extension of this type of surgery to the human arterial system, adrenal and cornea has been discussed, many other uses come to mind.

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[Discussion of this paper was postponed until after the paper by Hufnagel, which follows (See p 206)]

EXPERIMENTAL AND CLINICAL OBSERVATIONS ON THE TRANSPLANTATION OF BLOOD VESSELS*†

CHARLES A. HUFNAGEL

THE concept of homologous transplantation of vascular tissue is not new Like the general problem of transplantation of other tissue, it has intrigued man since the advent of the aseptic era of surgery Carrel (1907, 1908, 1910) in his epic work on the suture of blood vessels gave conclusive experimental evidence that homologous vascular grafting was practical. However, in spite of this and the work of Guthrie (1912), clinical application of this method was neglected until recent years and little progress was made

In 1944 we were stimulated by our work with kidney transplants and with coarctation of the aorta to reinvestigate the problem of arterial transplantation It was quite apparent that for such a method to be entirely practical on a large scale it would be necessary to use either homologous or heterologous transplants. It was also obvious that autogenous arterial segments were not available If venous segments were to be used to replace the damaged or resected artery, suitable autogenous venous segments would not be available from the same individual in the case of large arteries

It was apparent that some method of preservation must be available in order to have such grafts at hand for either emergency or elective use. We began our work with a survey of the methods employing tissue culture-like media It was found that satisfactory homo-transplants could be made after storage for relatively short periods with such solutions at

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temperatures just above freezing. But the limited storage life of vessels kept in these solutions made their replacement necessary after intervals of three to four weeks This did not seem an entirely practical technique Therefore, later in 1944 we began our work with rapidly frozen arterial segments (Hufnagel, 1947) Following this Gross *et al.* (1949) reported the use of a modified salt solution with 10 per cent serum as the storage medium for arterial grafts. This method has been rather widely employed, but has the inherent disadvantage of the short storage time which is permissible The necessity for uniform temperatures of storage and the large numbers of cadaver donors which are necessary to maintain a vascular bank with the method of modified salt solution storage has certainly kept it from being generally employed

Ideally one would like to set up certain conditions for the maintenance of large quantities of arterial segments in a vascular bank under the simplest possible conditions These general criteria would be as follows (1) Readily available arterial segments in suitable sizes (preferably not from a human donor) (2) The elimination of the need for aseptic technique in obtaining such grafts That is, a simple method of sterilization of the graft after it has been taken without impairing the function of the graft (3) Simplicity of storage There should be no necessity for storing in a solution which has to be changed nor of maintaining the graft at any constant temperature The temperature of storage should preferably be a noncritical environmental temperature (4) The procedure for preparation of the graft for storage should not be complicated. (5) The graft should have a minimal rate of thrombosis or breakdown and maximal functional capacity of carrying blood without aneurysm formation after transplantation

We have endeavoured in our research programme to fulfil these conditions so that vascular grafts might be available at any time and at any place It has been our feeling that the necessity for the use of human donor material would always greatly curtail the use of grafts because of the lack of the

availability of suitable young infection-free human donors This then has led us to the problem of hetero-transplantation. However, it was only after the good results obtained using homologous grafts that we have extended our studies further.

Rapidly Frozen Arterial Grafts

It has been our conviction that properly frozen arterial homografts could function satisfactorily in the replacement of major arterial segments, and such grafts have the distinct advantage of long-term storage. We have demonstrated that some elements of such rapidly frozen grafts survive the freezing process and can be grown in tissue culture. However, we are convinced that when such grafts are transplanted into another individual of the same species that the more specialized elements of the graft do not usually survive. Rather the tissues of the host use the graft as a framework and grow into the graft in a regular pattern, replacing to a large extent the original elements of the graft This is true whether the graft is a fresh homo-transplant or whether it is a stored homograft It should be pointed out here that the method of tissue culture evaluation of arterial grafts which are to be transplanted offers no evidence of survival in the host The fact that this tissue grows in tissue culture does *not* mean that the tissue will grow in the new environmental conditions after transplantation to another individual of the same species.

Our own research on several endocrine tissues and blood vessels and some of the work which has been presented here all bear out the conclusion that under the proper conditions of freezing, tissue can survive when transplanted back into the same animal. It should also be pointed out that when one transplants a blood vessel one is not transplanting merely cellular material which can perform its function essentially as a group of isolated cells, as does endocrine tissue, but that one is actually transplanting an organ which for its proper function must maintain its characteristics of structural integrity. It is

also essential that it must continue its function for years in order to be a satisfactory transplant.

The methods of rapid freezing which we have employed are described elsewhere in detail. Essentially, we have employed the freezing of tissue either in a bath of carbon dioxide ether or liquid nitrogen (Hufnagel, 1947, 1952). Both methods used properly have been satisfactory. We have regularly employed a storage temperature of —70°C All efforts are made to maintain this temperature as uniformly as possible once the grafts have been frozen. Fluctuating environmental temperature even below —40°C would appear to have a deleterious effect upon the tissue. Experimentally such frozen material has been employed in more than 300 animal experiments. The results must be classified to some degree according to the size of the vessel. When such grafts are over 6 mm. in diameter one may expect uniform success, the percentage being approximately between 90 and 95 per cent. When short grafts of smaller diameter are employed the percentage is less, averaging about 60 to 75 per cent. This figure, however, includes a number of very early experiments in which the sterility of the small grafts was not certain In more recent work, however, the small grafts have done better and are approximately 80 per cent successful.

During the last eight years such grafts have been employed experimentally in bridging defects of the thoracic aorta, abdominal aorta, pulmonary artery, iliac artery, and carotid artery of dogs They also have been used to produce shunts between the left ventricle and the aorta and between the right ventricle and the pulmonary artery (Donovan, Hufnagel, Eastcott, 1952) We have been impressed with the uniform success of these methods.

The histological changes are quite characteristic, and include a rapid degeneration of the muscle of the media with hyalinization of this area of the graft The endothelium grows across the transplant from each end This sometimes requires several months to bridge the mid-portion of the long graft The subendothelial tissue becomes thickened, particularly

the junction of the graft with the host artery. Fibrous ssue bridges the suture line within two weeks. New cells of pparently fibrous tissue origin grow into the graft both from e host artery and from the exterior where the graft is in ntact with the host tissue. In some cases there is a con- lerable thinning of the wall of the graft during the first onths after transplantation due to condensation of the edia. This has not appeared to affect the general function the graft and in our experience has not led to aneurysm rmation or excessive dilatation of the vessel The elastic sue of the aorta becomes condensed into a thick layer in e media The internal elastic membrane is usually intact d can be readily distinguished Such grafts have been served in animals now for periods up to five years with sentially no change from that which is observed in the first ar. At the end of this time stabilization has apparently curred and no further histological change is demonstrable. Frozen homologous human arteries have been employed nically in the thoracic aorta, the abdominal aorta, the iliac eries, and the femoral arteries Some of these grafts have w been in place for more than two years. In no instance s there been either thrombosis or disruption of the graft, d no aneurysm formation has been observed. These grafts ve varied in length from 10 cm. in the thoracic aorta, to cm in the iliac arteries Peripheral grafts have been made some cases in patients with severe arteriosclerotic oblitera- e disease, but in spite of this no difficulty has been en- intered in suturing the grafts, and their function has been cellent

In an effort to simplify further the problem of preservation vessels, following the work of Marrangoni and Cecchini 951), and Hyatt, Pate and colleagues, we have used the thod of freeze-drying In this case we are certain that grafts which are so employed are completely non-viable, d in this sense parallel our earlier work with grafts treated h formalin and other antiseptic and tissue preservative utions. The method of freeze-drying offers definite

advantages from the point of view of convenience of storage for long periods of time Dr. Pate has reviewed in his paper the significance of electrical potentials of the transplant, these would appear to play an important part in the process of intravascular clotting.

We have employed a simplified freeze-drying method which can be made readily available at low cost. This method employs a high vacuum pump with a mercury diffusion attachment. Specially designed traps, which are maintained at −190°C. with liquid nitrogen, condense the moisture as it is liberated from the tissue. The vessels are taken in the usual fashion and then are rapidly frozen, using liquid nitrogen or carbon dioxide-ether mixtures. While they are still in the frozen state they are packed in solid carbon dioxide and placed on the pumping mechanism Evacuation of the system is begun After two hours the solid carbon dioxide is removed from outside the vessel containers and the vessels are then allowed to reach room temperature Processing requires approximately eight hours, depending on the size of the vessels and the number of vessels in the system. During the latter part of this time the pressure is held to less than ten micra. At the end of this period the glass tubes in which the vessels are held are sealed with an oxygen-gas torch and the vessels are stored at room temperature. When the vial is broken the vessels are reconstituted in a balanced salt solution or distilled water, containing 1000 units of penicillin per ml The arteries reach a normal consistency in approximately fifteen to twenty minutes. They can then be sutured in place. Twelve such grafts have been placed in patients at the National Naval Medical Center in Bethesda, Maryland, with the co-operation of the Tissue Bank team operating there These grafts have been placed in numerous sites, ranging from the thoracic aorta to the superficial femoral artery In the twelve grafts there has been one thrombosis which occurred, not in the graft, but at a site just proximal and just distal to it in the patient's own artery It is interesting to note that this particular vessel had been

placed from a point just distal to the bifurcation of the aorta into the distal end of the common femoral artery. In order to bridge this defect two freeze-dried grafts had been sutured together. Several months following operation the blood supply to the limb was found to be diminishing and a secondary operation was carried out. At this procedure a thrombosis was found above and below the graft, but the graft, in spite of having no blood flowing through it, was still patent. There was no thrombosis in the secondary suture line. In this patient a new opening was made into the abdominal aorta just proximal to the bifurcation and a new graft was placed from there into the superficial femoral artery. This necessitated a graft which in its unstretched length was 35 cm in length and when it was in place was approximately 45 cm. in length. Following operation the patient again had pedal and posterior tibial pulses which have been maintained. From these experiences it has been our impression that all patients with obliterative arteriosclerotic disease should be investigated to determine whether grafting can reconstruct their vessels to maintain patency for long periods of time. In the series of patients in whom this has been carried out for arteriosclerotic disease we have had to date only two failures and feel that there is a very large group of patients in whom such procedures may offer long years of useful life.

To obtain arterial grafts aseptically from the postmortem cadaver offers a considerable problem If these vessels could be taken without aseptic technique the difficulties would be considerably reduced In an effort to do this a series of experiments was undertaken, utilizing cathode ray irradiation to sterilize the tissue Vessels were taken, rapidly frozen and then subjected to cathode ray irradiation. When irradiation dosage is sufficiently high to ensure sterility certain undesirable changes occur in the graft. However, lower dosage of cathode rays appear to exert no harmful influence on the graft from the functional point of view. It has been recommended therefore by some (Deterling *et al.*, personal communication) that to exclude the possibility of contamination of

grafts which are taken under aseptic technique small doses of cathode ray irradiation may be valuable. This does not in any sense solve the problem of obtaining grafts without all the surgical precautions essential in asepsis We have therefore employed a number of other materials in an effort to sterilize grafts without deleterious effects.

The use of organic gases with antiseptic properties has been extensively investigated. We have now employed ethylene oxide in both its gaseous and liquid forms for the sterilization of such grafts Sterilization by this method is reliable When used as a gas ethylene oxide may be used in a mixture with carbon dioxide, commercially available under the name of "Carboxide", or as pure ethylene oxide. It may be employed immediately after the grafts are taken or even more easily after grafts are freeze-dried. The gas can be admitted to the already evacuated chamber at the conclusion of the freeze-drying process so that the sterility of the entire apparatus can be further assured by contact with the gas. Any contamination which occurs during processing can thus be eliminated

The use of ethylene oxide as a liquid has certain advantages. The time of exposure necessary to complete sterilization is somewhat shorter because of the high concentration of the sterilizing material Liquid ethylene oxide has a dehydrating effect upon the tissue which does not appear to be deleterious Ethylene oxide is also a fat solvent, and the possibility of its action in the removal of certain fatty compounds may be valuable The work of Pate (1952) and of Sawyer and Pate (1953) has demonstrated that the antigenicity of tissue is changed by freeze-drying and we feel the use of ethylene oxide may further diminish the antigenicity of tissue. We do not as yet have data sufficiently conclusive to evaluate the mechanisms involved, but the experimental results in 30 animals parallel the results with grafts taken aseptically. Because of our success with this method in animal experiments it has been employed with human homografts, but it is too early to evaluate the results.

The lack of extensive peri-arterial tissue reaction in homografts which have been freeze-dried suggested that heterografting should be investigated. We have employed freeze-dried calf, lamb, and pig vessels experimentally. The results are quite different from those of fresh heterologous transplants. In the fresh state the success of heterologous arterial grafts is quite variable, but failure of the graft is to be expected in almost all cases. After freeze-drying and treating with ethylene oxide we have had no complications in 85 per cent of the animals In our early experiments there were several notable failures with disruption and disintegration of the graft. The treatment in these cases has been reviewed and we feel that the method of processing the graft in these cases was inadequate, and that drying had not been fully accomplished In the cases which have had what we now consider to be an adequate freeze-drying technique we have had excellent results. The tissues following implantation closely resemble those of homografts except that the peri-arterial response is more marked

We have been sufficiently impressed with this method that we have now successfully employed heterografts in two patients. In these cases the arterial grafts were obtained in an unsterile state In one case the vessel was taken from a calf and in the other from a young pig. The grafts were treated with liquid ethylene oxide for forty-five minutes They were then frozen and dehydrated, sealed in a vacuum of ten micra and stored three months and two months respectively. One of the grafts was used to replace the common iliac artery The origin of the right common iliac artery had been completely obliterated by arteriosclerotic disease and a new opening was made higher in the aorta and an end-to-side anastomosis of the graft was made to the aorta. Because the graft was too short to bridge the entire length to the end of the inguinal ligament, a second graft was sutured to the first. An anastomosis of the graft to the common femoral artery was performed just under the inguinal ligament Pulses have returned below the groin where they

were absent prior to operation The patient's foot is much warmer and oscillometric readings are now greater on the operative side than on the normal side, whereas they were absent prior to operation. The potential applications of heterologous grafts taken without aseptic precautions, sterilized with ethylene oxide, rapidly frozen, dehydrated, and stored at room temperature in high vacuum would appear to be the most practical of the methods which have been employed to date for the large-scale availability and the long-term storage of grafts. It is our feeling from the experimental data which have been accumulated that these grafts can be kept for very long periods of time; the exact time has not as yet been determined, but the storage period should be considerably more than one year if the processing has been carried out adequately. Other experimental work is now in progress in which the grafts are stored in an atmosphere of an inert gas instead of being stored in a high vacuum. In this process helium or nitrogen is admitted to the tube after dehydration. This makes the sealing of the tube considerably easier and we feel that this will probably be as satisfactory as vacuum storage.

At the present time it may therefore be stated that there is a simple readily available process for the rapid freeze-drying of arterial grafts Arterial homografts have been used in patients with good results The situations for which they have been employed include congenital obstructive lesions, such as coarctation, as well as aneurysms, trauma and arteriosclerotic lesions of the obstructive type. A new method of sterilization of tissue with minimal tissue change has been described, employing gaseous or liquid ethylene oxide

It would appear that such methods of sterilization when combined with a freeze-drying technique favourably influence the reception of the implanted tissue to such a degree as to offer a real hope that arterial replacement can be readily available on a large scale

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DISCUSSION

OUDOT* I have not used any freeze-dried grafts, but I have used in humans 80 grafts prepared by Gross's procedure I think my grafts are non-viable, because in four or six hours' time, I have never seen a growth in tissue culture My results are similar to those of Hufnagel and Pate In the 80 grafts, there were only three leakages, I had a leakage when I did a second graft on the same patient, and I think that in a case like this it is a question of an immunological reaction. I have also used grafts for bifurcation of the aorta, and I have now 12 cases with 10 good results The method of Hufnagel is very interesting and very convenient, and I am sure there is no immunological reaction

PARKES Do you know the residual water content of your freeze-dried grafts, Dr. Hufnagel?

HUFNAGEL. We have not measured that extensively Dr. Pate has done some work on that

PATE It is less than 1 per cent—about 0 8 per cent

EVANS Dr Hufnagel's statement, that viability of tissue doesn't necessarily mean that you're going to have a functioning graft, is very important I think it might be taken into consideration regarding the use of tissue culture as a means of determining if skin is viable. You've got to be very sure when you have a positive result that it is not merely

*(Editor's Note Dr Oudot died before he could check his part in this and subsequent discussions His comments have been omitted wherever the transcriptions left some point in doubt)

migration or unfolding of the cells from the edge of the explant, you have to be positive that it's real growth In addition it may not be wise, except and even in the hands of the very experienced tissue culturist, to extrapolate results either negative or positive into the final results to be expected from functioning grafts *in vivo*

ROGERS Dr Hufnagel, how long a follow-up period do you have on your heterografted arteries now?

HUFNAGEL The heterografts are quite recent, being about two months since implantation

ROGERS And have any of your patients died, not because of the operation, but concomitantly, so that they could be autopsied?

HUFNAGEL Fortunately not

PATE We have a series of freeze-dried heterografts in dogs which have been followed for about eighteen months—we have autopsied about 20 of them—and the graft looked fairly good both microscopically and grossly They do show an increased amount of fat in the media but not in the intima, which we can't explain at the present time

MEDAWAR Have any of the dogs which have been grafted with freeze-dried heterografts ever had second grafts from the same species? I ask that question because there might be a catch in the use of freeze-dried heterologous grafts Gibson has done some unpublished experiments on the use of boiled beef cartilage in human plastic surgery, and finds that although the first graft works fairly well the second ones sometimes do suffer an accelerated reaction of some sort

PATE We would use a different donor species the second time.

STRONG We have a case in the Tissue Bank file who has had four grafts to his nose the first one was a merthiolate cartilage which failed, that was followed by his own iliac bone, which dissolved, this was followed by a piece of frozen bone, and that dissolved, and that by a piece of his own cartilage which dissolved The last word we had from him was that he had had enough of grafting

ROGERS I think American plastic surgeons are very interested in the recent newspaper reports of the use of bovine cartilage in Great Britain for nose and ear reconstruction in the human I would be particularly interested in knowing what type of follow-up studies have been made on such bovine heterografts

EASTCOTT I was recently privileged to see a case of Sir Harold Gillies' in which he inserted bovine heterografts a long time ago He says that these bovine heterografts, although they reconstruct perfectly at the time and are tolerated very well in the early stage, undergo slow and steady resorption and some may need replacement, although I gather he hadn't met the problem of immunity response to the original species

MEDAWAR May I ask Prof Pomerat what circulatory cells he thinks could possibly re-form a vascular endothelium?

POMERAT It seems very difficult to see how the circulatory elements can come in and replace vascular linings along the new course of the graft On the basis of other information it seems much more likely the growth is inwards I would have liked to have seen the slides Mr

Eastcott showed at higher power to see whether this was a matter of œdema or what the cellular configuration was at the mid-point.

ROB. We have other slides which indicate that we get lamellar clotting, a thin clot on the surface of the graft, and certain cells in that clot produce a layer which resembles vascular endothelium

POMERAT That's a very interesting possibility, because there have always been those who have believed that monocytes have macrophagic capacity, and the Maximow Bloom school has maintained that even the small lymphocyte may produce a macrophage, and so in the chain of transformations one might get back as far as mesenchymatous elements with wide potentialities I think the field deserves a great deal of study

SANDERS This raises the whole problem of to what extent the tissues of one individual may be able to copy the contents of a foreign transplant placed within that individual When we look at the end results of transplants without studying intermediate stages, we may perhaps sometimes be deceived by the normal-looking appearance of the graft, it may, in fact, consist mostly of host tissue which has copied, or whose cells have reinhabited the matrix of the transplanted tissue.

POMERAT It has long been known that reverse transplantation of skin from the belly and back of the tadpole results in colour changes in the transplant Eventually the transplanted tissue is entirely replaced by adjacent cells I think in the case of the dead vascular segment, we are providing the organism with a temporary scaffolding which can be replaced from a cellular point of view and apparently eventually at the molecular level

ROB I should like to stress again this matter of delayed autografts of glandular tissue that Mr Eastcott mentioned briefly. I think it may have an important clinical application in selected cases, and that surgeons should consider very carefully the possibility of storing away human endocrine tissue and putting it back in graduated doses in accessible sites at future dates, and perhaps if one puts too much back, taking a little out again and so on It should be simple to do and may have a big future.

PARKES. Are you thinking of endocrine tissues only?

ROB There might be a case for other tissues, but I think the endocrines are particularly suitable In the case of an organ the problem of preservation is much more difficult if one's not preserving it in slices and then one would have to reconstitute the blood supply subsequently With the thin slices of endocrine tissue, just like the skin graft, you hope to get the blood supply to the surface

PARKES Have you any positive results yet?

ROB No. We have been storing human adrenals and other tissues for just over a year, and we have not yet got a really deficient patient for such a delayed autograft In one patient whose adrenals were removed for Cushing's Syndrome over a year ago we may need a graft soon and we have kept his adrenals in glycerol saline at −70°C ever since

JONES I don't want to be discouraging, but I've done a certain

number of experiments and Dr. Smith and Dr. Parkes have done many more, on storing adrenals at −79°. I believe they have been unsuccessful and I've had no success at all. At −79° it seems doubtful whether adrenals, anyway, remain viable for more than twenty-four hours—if that.

ROB. Ovarian tissue does.

PARKES. In the medium we have been using, −79° is not a very good temperature for preservation, but we think that is mainly a matter of further work on the medium. From the work that we've done in the rat, it seems that ovarian tissue lasts better at −79°C if it is frozen in glycerol serum rather than glycerol saline. There's a great deal more work to be done on the question of medium as well as on temperature. But have you any experience with human adrenals stored in glycerol, Mr. Jones?

JONES. No. Only of the embryonic graft in rats.

PARKES. This may well be another case where it's not safe to argue from rat to man.

BILLINGHAM. Mr. Eastcott, you mentioned very briefly an experiment on corneal storage. Were you referring to experimental work or to the clinical application of a graft?

EASTCOTT. Both. These were experimental operations conducted with a new method in man. They were justified by the fact that they were lamellar grafts which could quite easily be repeated without detriment to the patient's condition, and no fresh material was available.

THE PRESENT STATE OF CORNEAL GRAFTS

B W RYCROFT

BEFORE we pass on to the consideration of factors which influence corneal grafts, either for good or evil, it is essential that we should clearly evaluate our standards and decide what is to be the yardstick of success in a corneal graft.

There is some confusion in international circles as to the method of assessment of the results of corneal graft operations Some authorities such as Tudor Thomas claim that the anatomical clarity of the graft rather than the improvement of vision should be the standard They argue that vision can be influenced in graft cases by factors unpredictable before operation such as early cataract and retinal diseases. They state, with truth, that the pathology of the posterior eye cannot always be diagnosed before a projected graft operation owing to the extent of the corneal scar. Yet others like Stansbury prefer a physiological basis and measure their success by the improvement of vision obtained These differences in the method of assessment have bedevilled comparison of the international results of graft surgery since anatomical transparency does not necessarily mean improvement of vision Today, however, as we are discussing grafts of many kinds which are based on anatomical success the same standard will be applied to corneal grafts. It should, however, be remembered that the ophthalmic surgeon demands high standards for his grafts: not only must the graft "take" and remain permanently transparent but it must also conform to the regular radius of curvatures in the host if clear undistorted vision is to be obtained. For example, a clear graft with distorted curvatures may not improve vision on account of irregular astigmatism.

Corneal grafts today are used for four main purposes·—

1 Restoration of vision by the replacement of opaque scar tissue (optical). 2. Acceleration of healing by the coverage of an indolent corneal ulcer by fresh graft tissue, which is the same principle as is used for cross leg flaps and varicose ulceration (therapeutic) 3 Corneal grafts are also used for the sake of appearance, to replace the hideous white staring scar of a blind eye (cosmetic), and 4. They may also be preparatory, where there is a marked disparity in the thickness of the host cornea compared with that of the graft (preparatory) Today only the optical grafts will be discussed although from the immunological aspect therapeutic grafts present many interesting problems.

In technique there are today three types of graft, namely·—1 the full thickness type, which can be compared to the Wolfe skin graft, 2. the split thickness type, comparable to the Thiersch skin graft; and 3. the flange graft, which is our own method, or as it is called by Franceschetti, the "mushroom" type. Full thickness grafts must be used where the corneal scar involves the whole thickness of the cornea. It opens the eye ball and is therefore liable to complications About 30 per cent of these cases retain anatomical transparency. In the split or lamellar type of graft complications are few, since the eye ball is not opened, but there are severe limitations and only superficial scars are amenable to this method On the other hand, about 70 per cent of split corneal grafts remain clear. The flange type of graft has been evolved to avoid the complications of the full thickness method The graft is the shape of a back collar stud and is composed of partial and full thickness parts. It aims at providing a self sealing section and avoids many of the complications of the full thickness method

The factors which influence optical corneal grafts are —1 technique; 2 infection; 3. selection of cases; 4. selection and preservation of donor material; 5. vascularization, 6 œdema

Technique need not be discussed in detail today, but it is probably of more importance in corneal graft surgery than it

is for other types of grafts since these in general conform to well established principles of surgery. To a certain extent technique has varied only in detail in the last fifty years New and fine-precision instruments have naturally changed details of surgery a little but the basic principles of accurate fixation and strict asepsis remain In the penetration method the object must always be to seal the wound in the eye ball quickly to avoid the possibility of inclusion of iris or other intraocular contents in a leaking section, and in the non-penetration methods absolute fixation of the graft is also essential. Nowadays, direct edge-to-edge sutures and appositional cross sutures are the main methods of fixation. It is vital that needles for suture work and all cutting edges should be exquisitely perfect. In the East Grinstead clinic cutting edges and points are all subject to microscopic scrutiny by the slit lamp before operation.

Infection is no longer a great problem in corneal graft surgery and in our last 100 cases it has not been of any serious consequence. Two types have been encountered· one is where there have been coincident severe burns of the face and conjunctiva with considerable chronic lid infection and the other has occurred as a small residual abscess in the graft bed. This was a feature in some of our early cases and was probably due to aerial contamination since granules of talc, etc. have been found behind the graft All usual pre-operative investigations and selective antibiotics are used· for *Bacillus pyocyaneus*, which is so common in burns cases, we have found local polymyxin very satisfactory. Culture of the donor eye is also essential

The condition of the host cornea has also an influence on the eventual success of the graft In general it may be said that if a graft is completely surrounded by dense fibrous tissue it is unlikely to survive, so that if possible grafts should always be planned to abutt on normal scar tissue. It will thus be seen that in experimental animals if any accurate comparison is to be made the graft must be placed in scar tissue and not in the normal cornea of the animal. Also a

graft should not be carried out on a scar which is still vascularized from the initial injury, the quieter the scar the better the chances of avoiding graft vascularization

The type of host eye disease which has produced the scar is of consequence. The scars of conical cornea and interstitial keratitis are generally successful but the dense scars of burns from war injuries unfortunately are not Acne rosacea and leprosy have both been known to spread from the host after the graft has been successfully performed

The donor problem is still with us. The latter half of the 19th century could be called the phase of heteroplasty when there was a plethora of dismal failures in corneal graft surgery Now although these have been put down to the fact that these grafts were heteroplastic it is also very likely that severe infection, immature technique and inefficient instruments contributed to these failures. There was also poor anæsthesia and a confusion of ideas on grafting. Slowly the fires of enthusiasm died away but there still remained a few glowing embers The first heteroplastic graft into the human was made in America by Kissam in 1838. He used the cornea of a six months old pig and the eye of a man blinded by a dense central scar Vision immediately improved but within a fortnight the graft became opaque and in a month it had merged in to the original scar tissue. In 1886 and 1887 Von Hippel exhibited a patient at meetings of the German Ophthalmological Society at Heidelberg where after four unsuccessful attempts he had succeeded in implanting a full thickness corneal graft from a rabbit into a partial thickness bed in a human Vision was improved and must have lasted at any rate for a year But it fell to Zirm in 1905 to apply a sharp draught to the smouldering fires by the report of his famous case of human transplantation which restored the sight of a man blinded by lime. Heteroplasty was abandoned and when homoplasty became the fashion corneal graft surgery in Europe rapidly expanded. Yet we must not entirely dismiss heteroplasty, since last year Babel, Bourquin and Choyce have again reported successful corneal heteroplastic

grafts in animals Experimental evidence of this kind is valuable in that it will enable us to study the behaviour of heteroplasts but it is unlikely to influence the general use of homoplasts. It is interesting to note in Babel and Bourquin's series that the tolerance of heterogenous tissue did not appear to be peculiar to a given species nor was it related to any particular part of the donor cornea. Rabbits tolerated well grafts from ox, horse, pig and sheep Also it is of importance to note that a second graft from the same and from a different species was not tolerated as well and nine such grafts all rapidly became opaque although the clarity of the original graft was not affected One cannot help feeling that this is parallel to the work of Medawar on rabbit skin grafts There is no evidence to show how these grafts would behave if they had been preserved. they were all fresh grafts. Inorganic hetero-transplants must be briefly mentioned and in doing so one may say that the wheel of fashion has turned a full circle. The original idea of inserting crystal into a scarred cornea came in 1778 from de Quengsy; Franceschetti and others have recently tried plastic corneal implantations with varying success In our own cases the implant was discarded and even if it had remained fibrosis would have impaired vision.

Von Hippel in 1887 founded the basis of homoplastic keratoplasty. Eyes were removed much more frequently in olden days than they are today and there was probably an adequate supply of donor material. Nowadays, however, the only source of fresh material is from eyes which have to be excised for tumour of the posterior eye, such as sarcoma, or from degenerate eyes of absolute glaucoma, occasionally an injured eye is suitable. Only once has the original neoplastic disease of the donor occurred in the graft this was from a case of glioma and was reported by Kato in Tokyo.

But there is no doubt that this supply of fresh grafts is steadily diminishing as more intraocular tumours are successfully treated by radium, radon and possibly by isotopes. In any case, a cornea removed from a pathological eye is not

ideal since the eye has often been under tension prior to excision but by force of necessity this source has had to serve in the past. In 1922 Filatov reported the use of cadaver cornea which had been preserved in an ice box and he preferred preserved corneas to fresh material.

The ideal donor is the cornea of an aged individual who has died a natural death the cornea of the fœtus is too pliable and sharply curved There is no doubt that it is to the cadaver cornea that we must look in the future for our main source of donor supplies, methods of preservation, therefore, are of the highest importance At one time it was thought that a cornea or eye should be removed from the cadaver within two hours of death but now it is found that fifteen hours post mortem is probably the limit. Once it was found that this longer period of time was feasible administration difficulties of collection were easier to overcome. The length of preservation has been as long as three weeks for optical grafts and many methods are used. Paufique favours saline vapour, Tudor Thomas saline or a moist chamber, Barraquer in a dry test tube on ice, and Feldman by partial dehydration At East Grinstead, Burki's method is used by which the cornea is preserved in sterile liquid paraffin at 4°C ; such grafts have been successful after three weeks of preservation. During that time the cornea is known to consume oxygen. Douane reported that respiration is maintained in oil but is reduced by 70 per cent by freezing to −40°C and is abolished when the graft is preserved in formalin For our purpose the viscosity of the liquid paraffin is also valuable because so many of the donors are transported by rail and the inertia of the oil protects the eye in transport. Before use at operation the oil is removed from the donor by washing in 1 per cent Cetavlon and spraying with penicillin saline solution. The graft is never immersed in aqueous fluid as an alteration in thickness would occur, it is retained in moist penicillin gauze.

However, until recently it has been quite illegal for people in this country either to bequeath their eyes for surgical purposes or for any surgeon to remove any organ from the dead

body except on the coroner's instructions Since there are a large number of enlightened people in this country who wish to help research in the cure of blindness it seemed, therefore, that steps should be taken to abolish restrictions which prevented the bequest of eyes and their post-mortem removal. Accordingly, in May, 1952 a Bill was presented to Parliament with this object in view and it became law in September, 1952. It is now legal for a surgeon to remove eyes for therapeutic purposes from a dead body provided no objection is raised by the nearest relative. It is also legal for people to bequeath their eyes and from personal experience I have to report that near relatives have never objected either to personal request or to the removal of eyes from bequest. The natural sequence to this Act has been the establishment of Eye Banks throughout the country The Bank at East Grinstead has received a considerable increase of material since the passage of the Act and frequently a team from the hospital has gone out and collected eyes within a few hours of death. It is of significance to note that on such an unusual occasion the nearest relative generally treats the bequest of the deceased and the whole procedure with a sense of dignity and duty. The problem of donors in this country has therefore been much diminished by the Corneal Grafting Act of 1952.

The risks of infection and the difficulties in the supply of instruments and donors which confronted us at the end of the last war have been largely overcome. The problems which remain lie, I think, in the province of the laboratory rather than in the operating theatre. They are those of graft vascularization and œdema.

Vascularization of a corneal graft may take three forms: Firstly there is the superficial traumatic vascularization which immediately occurs after operation and which generally subsides. Sometimes, however, it persists and invades the graft, with resultant fibroplasia Then there may be the aggravation of existing blood vessels in the host cornea which may invade the graft to all depths and bring about opacification. Thirdly, there is the late vascularization which occurs

towards the end of the third week and is generally accompanied by œdema Previous attempts to control this vascularization have been by beta-radiation to host and donor, retro-bulbar injections of alcohol, and by cortisone At East Grinstead no definite conclusion as to the effects of radiation have been reached but there is no doubt as to the value of local cortisone Not only does cortisone reduce irritation and discomfort but it also, according to Ashton, prevents the endothelium buds from growing out of new blood vessels, and new vascularization is, therefore, clinically controlled to a great extent It has no effect on established blood vessels. If the cortisone is given parenterally adverse results are seen and delayed healing of the graft occurred in two of our early cases.

Oedema of the cornea is a problem which is ill understood. It may involve both full thickness or lamellar grafts and it generally occurs in conjunction with vascularization. Sometimes it suddenly comes on about the third week in a graft which has previously been clear and satisfactory (Maladie du greffon). Hypertonic salines have some transient effect on this œdema but the ultimate outlook is gloomy. Cortisone has very little effect and œdema, therefore, remains the dominant problem at the present time It is hoped that perhaps you may be able to help me with a discussion of the problems of immunology in relation to corneal grafts. Total corneal grafts with a scleral frill have been attempted, but so far results have been entirely unsuccessful except to establish a method of technique These total grafts were performed on volunteers and on blind eyes but in every case the eye has had to be removed subsequently To my mind, however, if we can solve the problem of why these grafts fail (and there was no evidence of infection) it will lead us a long way to solving other problems of corneal graft surgery and to help a further proportion of cases which have hitherto been considered to be hopelessly blind

My feeling is that the improvement of results in corneal graft surgery rests not entirely in the improvement of tech-

nique but rather in a better understanding of the immunological problems which beset graft and donor.

DISCUSSION

BILLINGHAM I should like to mention briefly some experiments that Miss Boswell and I have carried out to determine whether corneal homografts are in fact antigenic as skin homografts are known to be We have found that when thin shavings of homologous cornea are transplanted to richly vascular beds prepared in the skin of a rabbit's chest, the grafts heal in, become vascularized and their epithelium proliferates just as it does in the case of corneal autografts so transplanted. However, the hosts did not long tolerate their heterotopic corneal homografts they broke down in the same manner as skin homografts Furthermore, once a host had reacted against corneal homografts it became so affected as to accelerate the destruction of further corneal grafts later transplanted from the same donor. It is inferred from these results that when corneal homografts are transplanted so that they become vascularized, they immunize their hosts and are therefore antigenically effective

We have also tried to shed some light on one aspect of a further problem Mr. Rycroft has referred to whether a corneal graft functions simply as a framework graft, or whether some of its cells may survive indefinitely We have investigated the effectiveness of transplantation immunity within the substance of the intact cornea by transplanting minute skin homografts to pockets cut in the corneas of rabbits which had previously been immunized against their intended donor's skin The animals were destroyed at various intervals after grafting and the fate of the grafts determined, either on the basis of histological examination, or by transplanting them back to the original donor from which they were taken as a biological test of their continued viability. We found that the skin grafts transplanted to the corneas long outlived skin homografts transplanted on the same occasion to the recipients' chests, provided that the grafts in the corneas remained unvascularized. If, however, vessels from the limbus grew out and penetrated the grafts, they promptly broke down From this it was inferred that tissue transplantation immunity is ineffective within the substance of the cornea so long as it remains avascular

These findings make it clear that cornea is not an "immunologically privileged" tissue, but a tissue in an immunologically privileged position. The question of whether homografts transplanted to the cornea can themselves provoke immunity is thus of no practical importance, since any such immunity would be quite ineffective so long as the grafts remained avascular.

RYCROFT As a clinician, two considerations occur to me. We would not regard a sliver of the cornea as a true graft—it is only part of the cornea And the environment of such a graft should be either in control or comparable—there should be aqueous humour on one side and air on the other before a clinician could draw conclusions.

Dempster I was interested in the bacterial pathogens. Would you say that pyocyanea is not common in diseases of the eye?

Rycroft It is extremely uncommon in the ordinary way, but a lot of my graft work on eyes is with a burns Unit Pyocyanea is almost a normal inhabitant of such conjunctiva, but the burn is the origin

Dempster It is interesting because A. B Wallace of Edinburgh finds pyocyanea frequently associated with the breakdown of skin homografts And in the kidney of the dog, one can quite frequently culture a scanty growth of pyocyanea in the homografted kidney when the immune response has come on and when the tubules look histologically satisfactory, but the kidney has stopped secreting I was wondering if you had tried terramycin locally in these cases? Terramycin seems to be the only available antibiotic which is effective against pyocyanea

Rycroft We haven't tried terramycin because we have found reasonable control with polymyxin

Dempster Is it a pure growth of pyocyanea? There's great doubt amongst some bacteriologists whether pyocyanea is really, in fact, pathogenic You don't get a mixed growth?

Rycroft The pre-operative culture investigation shows us consistently the pyocyanea in burn cases we treat them with the polymyxin first as a routine before corneal grafting

Dempster I see But when you get a breakdown, is that ever connected with pyocyanea infection establishing itself?

Rycroft No, conjunctival cultures in corneal graft breakdown are sterile

ing instructed in Tissue Bank technique. The Tissue Bank
ice and its civilian secretary are the administrative centre
the clinical research project and the follow-up system
At present our principal source of tissue is sterile post
ortems on patients that die in our hospital. To be used as
tissue donor the patient must be free from malignancy,
ectious or contagious disease, including syphilis, and
patitis. If these conditions are met, a medical officer from
e Tissue Bank talks to the next-of-kin to ask permission to
nove tissue for grafting. In occasional cases the physician
king care of the deceased has done this for us. This per-
ssion has been obtained by telegram following a long
stance telephone call to the relatives If this request is made
the next-of-kin in the proper manner by an individual who
well versed in the Preserved Tissue Program, it is seldom,
ever, refused This permission is always obtained in writing.
is must be done within twenty-four hours of death and the
dy must have been refrigerated in the interim (Deterling,
58).

The body is brought to the Tissue Bank work room where
e trunk and lower extremities are shaved and scrubbed with
detergent soap, containing hexachlorphene. The body is
oved to the special operating room, which has been previous-
prepared Standard operating room instrument tables are
up, sterile drapes and linens are provided in large quanti-
s. At the start of the procedure, the body is placed face
wn on the operating table, care being taken not to distort
face, or stain the hands and other exposed areas with
antiseptic solution used to prepare the skin.

The skin is prepared with merthiolate following an ether
an-up. The back, buttocks and posterior thighs are draped
h sterile sheets Large rectangular pieces of skin are taken,
ng the Brown electric dermatome. This instrument is fast
d efficient, permitting the removal of large areas of skin in
hort time. As each piece of skin is removed a record is made
its size and it is placed in a metal tray After the tray has
en filled, cultures are taken and the tissue is then liberally

soaked with a saline solution of penicillin and streptomycin containing 500 mg. per ml. of each of these antibiotics. The skin is quickly frozen by pouring an excess of liquid nitrogen into the tray. It is quickly double wrapped in sterile muslin wrappers, securely tied and immediately placed in dry ice storage at —76°C. to await freeze-drying. This technique of homogenous skin preservation is much more rapid than the nutrient media method we previously used (Hyatt, 1952). The latter is probably the method of choice for storing autogenous skin for reuse on the donor; however, freeze-drying produces a material that works as do other types of skin homografts and it has the advantage of longer storage. Next, all drapes are removed and the operating team is regowned and regloved, and the next operative area is prepared. Between each operative site and each new type of tissue fresh drapes and fresh instruments must be used to prevent cross contamination Skin towels are always used.

Tissues are removed in the following order. First, the skin of the back as described above. Second, the scapulas, following which the body is turned on its back, reprepared and redraped. Third, the femoral vessels are resected from the inguinal ligament to ten inches distally. Great care must be taken to divide all arterial branches at least ten millimetres from the main artery Fourth, the tibias and femurs are resected through a lateral incision by subperiosteal dissection. Lane no-touch-technique and the oscillating electric saws are used to remove the long bones. The ilia are removed next. The chest is then entered and the sternum and ribs are divided at the costochondral junction and the sternum removed, the thoracic aorta removed, as well as the third, fifth, seventh and ninth ribs on each side Lastly, the abdominal cavity is entered and the aorta and the iliacs are removed.

A word about arterial dissections as applied to graft procurement. It cannot be over emphasized that the utmost care must be used in dissection of arteries. Their branches must be divided at least 10 mm. from the main vessel. These branches must not be put under any undue tension which

would subject them to strain where they leave the aorta, as this would result in a tear which does not become evident until the graft is reconstituted This is particularly true of the intercostal branches of the aorta

Arterial grafts are washed in physiological saline, cultured, sprayed with antibiotic solution and assigned a number. They are then laid aside in a glass tube for freezing after all have been removed. We have been unsuccessful in preserving arterial grafts from donors over thirty years of age Over this age we do not even attempt arterial dissection because minimal arterial sclerosis results in cracking of the vessel during processing.

The elaborate aseptic ritual used for tissue procurement should be emphasized Each time we change an operative site or start the removal of a different tissue the operating team must change masks and rescrub prior to donning new sterile gowns and gloves In addition, fresh instruments and fresh drapes must be used for each tissue and each operative site. During the operative removal of tissue all participants wear double masks which are replaced with fresh ones every hour on the hour Also, all personnel in the operating room must wear muslin boots over their shoes No talking is allowed and hand signals are used for all communications. During the average autopsy, which requires twelve to fourteen hours to complete, between 200 and 250 cultures are taken. It has been our experience that if this elaborate aseptic ritual is *not* followed, contamination becomes progressively more frequent as more grafts are processed and cultured. This has resulted in as high as 20 per cent of the deposits of a given autopsy being rejected for contamination, principally with airborne bacteria This occurs in spite of two ultraviolet air sterilizing lights that are continually burning in the operating room. If, on the other hand, the strict aseptic ritual is rigidly followed, contamination is cut to 1 per cent or less Contaminated grafts are discarded.

After the last tissue is procured, the body is removed to the work room for reconstruction. The femoral vessels are

iulated with plastic tubes to facilitate embalming and
long bones are replaced with intermedullary wooden
els to secure proper length and alignment of the extremity.
skin incisions are closed with a continuous suture, and
body returned to the morgue for the pathologists to hold
outine necropsy If an abnormal or pathological condition
iticed during the sterile autopsy, we personally inform the
iologist. In spite of the extensive dissection we have
ied out, the pathology department has no difficulty in
lucting their normal post mortem examination. The only
cture we have disturbed in which they might be interested
ie aorta, and if this was abnormal, they receive a state-
t as to its condition

ollowing the removal of the body to the autopsy room,
ks are changed, clean drapes and instruments provided
the process of cleaning, cutting, grinding and culturing
bone is undertaken. Cartilage is separately processed.
s tubes containing arterial segments are carefully im-
sed in a solution of dry ice and alcohol at −76°C Care
t be taken that none of this alcohol comes in contact with
graft After ten minutes in this solution, the vessels are
ble wrapped with muslin wrappers, labelled and quickly
ed in the deep freeze to await desiccation

s they are cleaned, the ilia and scapulas are cut into pieces
ground into particles a little smaller than a grain of rice,
ig the Roger-Anderson Bone Mill Ribs are treated in a
ilar fashion or are split into "matchstick" grafts Approxi-
ely one ounce of this ground bone is placed in each bottle
r cultures have been taken The tibias and femurs are
into flat cortical grafts, cultured, placed in bottles,
iyed with antibiotic solution and temporarily capped.
h bottle has a metal band that is stamped with the control
iber. A sequential record of this number and the contents
he bottle is made in a large notebook and on a bacteriology
ure request slip This slip is sent to the laboratory with
material for aerobic and anaerobic cultures The bottles
one are placed in large trays, doubly wrapped in muslin,

labelled and stored in the deep freeze at —76°C., to freeze before vacuum drying.

If we were unable to reach some of the staff or if they were unable to come in at the time the post mortem was done, they report at this time to clean up. The procedure as described takes about five hampers of linen, including 75 pairs of gloves and glove wrappers, 50 gowns, 400 towels, 200 sheets and a host of miscellaneous material, including 500 wrappers Approximately 100 to 125 grafts of all types are obtained from the typical young cadaver.

The tissue is now ready for freeze-drying. We use a commercial freeze-dryer that has undergone extensive modification in our Tissue Bank This machine is essentially a large steel chamber that contains three hollow metal shelves The two bottom shelves are cooled to a temperature of —50°C by an internal system using "Freon" 12 as a refrigerant. These shelves serve as a condensing surface to trap the water vapour that has sublimed out of the tissue during the vacuum drying process The third or upper shelf on which the tissue is processed, is connected to a reservoir of trichlorethylene A small electric pump in this system permits us to circulate this material through the plate as a refrigerating or heating agent. When the freeze-drying cycle is started and we want to maintain the tissue in a frozen state, the trichlorethylene is cooled by a bath of dry ice and alcohol. This enables us to lower the temperature of the upper plate to approximately —50°C. This plate can also be warmed to supply the latent heat of sublimation by means of a thermostatically controlled electric heater in the trichlorethylene reservoir Thus the temperature of the processing plate can be accurately controlled. We have replaced all vacuum gaskets with a new ethylene polyfluoride polymer called "Teflon", which is an excellent high vacuum gasket material The machine is so designed that it can be sterilized with steam in the same manner as an autoclave. After sterilization it is cooled to room temperature prior to use

Our usual technique is to run the internal refrigeration

system for twenty-four hours before loading the machine for vacuum drying. The tissue is removed from the deep freeze and unwrapped, using sterile technique. By this time the bacteriology laboratory has reported on the cultures taken during the procurement and any contaminated material is segregated and removed before loading The trays of tissue are placed on the top shelf where they are held in a frozen state while subsequent trays are removed from the deep freeze, unwrapped and loaded This is all done as quickly as possible The door gasket is coated with high vacuum grease, the door closed and securely dogged down. The vacuum pump is immediately cut in and the chamber rapidly exhausted to a standing pressure of between 70 and 100 micra of mercury. A careful check is maintained on the temperatures and pressures during the drying cycle A written record as well as a visual graph is kept The upper shelf is allowed to rise gradually to 0°C as sublimation takes place On the fifth day there is approximately 10 per cent residual moisture in bone. The temperature of the upper shelf is then raised to 30°C., supplying the latent heat of sublimation to remove this water. At the end of fourteen days the cycle is terminated, the bone now containing less than one per cent residual moisture The material is brought to atmospheric pressure, removed from the freeze-dryer, and quickly vacuum packed, again under sterile technique, by using a small vacuum pump to pull the secondary vacuum through a hand capping unit.

In our equipment, bone requires fourteen days to dry However, blood vessels and skin are much more rapidly dried, requiring only three days for removal of 99 per cent of tissue fluids

We have experimented with many types of containers We are currently using the two sizes of small diameter Baxter bottles and have found them to be the most satisfactory of all types tested. They are commercially available and are much less expensive than any other type of unit we have used. In addition to the rubber stopper and metal cap a secondary seal

of hot wax is applied Of all the compounds tested the Apiezon, manufactured by a British electrical firm, is the most satisfactory for maintaining a good vacuum over long periods of shelf storage Following the capping and sealing, the material is labelled, multiple checks are run to make sure that no contaminated grafts have slipped through, that all records, numbers, measurements, and descriptions agree. The grafts are then placed on the shelf for storage at room temperature until called for by the surgeon.

A word about records. in running a project of this type, records can never be too detailed or too carefully kept Sufficient to say that we have learned the hard way Some of our records are· 1 A legal form that authorizes us to remove the tissue 2. A tissue deposit withdrawal card that is made out for each graft, giving its control number and describing in detail, who, where, when, what, how, and why. The results of the blood test for syphilis and the culture report from the bacteriology laboratory are also included. The reverse side of this form is equally extensive, covering all the information on the recipient of the graft. While the graft is in storage these cards are kept in chronological order The grafts are stored as to type, and when one is selected for a given case, the card is easily located, the vacuum is tested by ionization with a high frequency spark, the culture reports double checked and the graft prepared for use Arterial, cortical bone and freeze-dried skin grafts are reconstituted with a saline solution of antibiotics Ground cancellous bone is generally used dry without prior reconstitution

As the Tissue Bank is a clinical research project to evaluate grafts and grafting, one of its prime functions is the maintenance of careful, accurate and complete records on all patients receiving grafts The file on each patient includes a case summary, the operative record, the deposit and withdrawal card (which gives the history of the procurement, processing and storage of the graft), copies of photographs, X-rays, and special laboratory tests All bone graft patients are X-rayed every six months post-operatively and, wherever

possible, receive at the same time physical examination and evaluation. As this project is not limited to the Bethesda Medical Center, we have the co-operation of the other military medical services in obtaining follow-up studies. To date, we have been successful in maintaining follow-ups from every case receiving grafts from this Bank, but it is too early to allow definite conclusions to be drawn from this work.

This has been an outline of the manner of procuring, processing, and studying tissue preservation in the United States Navy. We feel that this is a new approach to this field in surgery. We hope that these first steps have been in the proper direction and that this pilot plant may provide a pattern for Tissue Banks in civilian and military medicine. Much of the information gathered at this symposium will aid in improving and refining our methods.

The opinions expressed here are those of the author and do not necessarily represent those of the Bureau of Medicine and Surgery or the Defense Department

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DISCUSSION

EASTCOTT I was very interested to hear that you have had trouble with cracking when freezing arteries from donors over 30 years of age. Hufnagel and I found that in freezing dogs' arteries with liquid nitrogen we experienced thermal cracking at a very low temperature, when the arteries had equilibrated at −195°C, but we never saw cracking when freezing arteries in dry ice mixture; and subsequently in dealing with human material we've never had cracking of that kind. You thought that arteriosclerosis was responsible, but with frozen arteriosclerotic vessels, I've not seen cracking result.

I note that a tremendous amount of bone and much skin was removed

and only a moderate yield of artery was obtained. How does this match the consumer demand for these three commodities?

STRONG We are engaged in a clinical research problem rather than a production effort We don't get nearly as much tissue as we could use We have many requests to help us evaluate this material, but we don't have the material to give away. Our hospital is not an ideal place for an endeavour of this type, because we don't have a large traumatic service Many of the people that die in our hospital are old retired veteran personnel, and particularly from the aspect of blood vessels they are unsatisfactory

HARRIS I am worried by the efficiency of the freeze-drying process It seems to me that these long periods of freeze-drying might be considerably shortened if more attention were paid to thermal contact between the material being dried and the source of heat, in the top shelf With a piece of bone standing in a bottle, standing on a tray, standing on a heated shelf (*in vacuo*) you have almost no thermal contact between the material and the source of heat. A re-design of that part of the apparatus might pay considerable dividends I suggest for example the possible use of metal containers, or the use of trays for your "boneburger"—metal trays can be capped in a sterile manner by a dropping metal lid, as some of the penicillin people have been doing

And the other point that occurred to me is this, why put your material on the top shelf, and condense your water on the bottom shelf? Would it not be better to raise your cooled surfaces, and have your water coming up out of the container on to the condensing surface? I realize that many of these difficulties are hard to overcome because of the design of commercial apparatus

STRONG We agree with everything you have said With commercial equipment, you take it or leave it, if you have special equipment built it runs up the cost considerably. We have been thinking about new freeze-driers There is now a small commercial freeze-drying unit that supplies heat by infra-red radiation It has been used in some bacteriological work, and it's my understanding that on drying with infra-red radiation they get a much higher yield as far as survival of their bacteria is concerned We have thought about applying that to our tissues However, we don't want to change horses in the middle of the stream, so to speak, on a research project, and for various limitations of plant size and other things we haven't gone into a new freeze-dryer

As to drying material in trays, our only objection to that is that we like to put the material in its final container in the operating room, and have a minimum of handling As to metal containers, we are currently working on putting grafts up in tin cans, and they will be processed in the cans, probably with better heat transmission

HARRIS One would like to see the sides of the can and the bone in fairly close proximity, so that you do get your heat in So far as I can see you're getting very little in at the moment.

STRONG We're getting very little, the vast majority of our drying is done by the vacuum rather than by supplying heat

HYATT I should like to emphasize that we are very careful not to mutilate the body, it must be cosmetically perfectly acceptable The Tissue Bank people are trained with special care given to decorum. The æsthetics and proprieties that go with the dignity of death are fully observed We have found that our difficulties are minimized by this approach

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PARKES I have the impression that this symposium has served a most useful purpose, particularly in bringing together, as Sir Harold Himsworth said at our Dinner last night, varieties of experts who in the ordinary way might never meet at all That was part of the object of the symposium, and it has, I think, been very well fulfilled Another thing which strikes me is that while many meetings of this kind are devoted largely to integrating the past, here we've been mapping the road to the future. A rather interesting observation which again distinguishes this meeting from many meetings of scientists, is that there's a very small proportion of grey heads among the membership This subject in its present form is a young subject and its exponents are mainly young people, and this seems to me to be highly desirable insofar as we are investigating among other things the potential immortality of stored tissue—if you want to do that, start young!

Of the discussion and papers we have had, what stands out most clearly in my mind is the very clear differentiation which has been brought out between tissue grafts which will be of value even if they are dead or dried—certainly if they have no viable cells—and those tissue grafts which depend entirely on functional survival of the cell for their effect I myself, and I think other laboratory workers here, have been quite astonished at the extensive clinical and surgical use which can now be made of tissue which is admittedly dead In the course of the discussion someone pointed out that where a graft depends for its value on structural integrity, then a dead graft might very well function adequately, on the other hand, where cellular function was necessary, then a live graft was of course essential This seems to me to be a very real distinction

The other thing in connection with the dead grafts which interested me very much was that the processing, particularly freeze-drying, might reduce the antigenic properties of the homograft so greatly as to make the dead homograft almost entirely acceptable to the host, and the extreme example of that we heard from Dr Hufnagel, where heterografts are also acceptable under certain conditions, at least for a time Many of us would like to hear later what has happened to these pieces of pig placed in man. The fact that the processing can reduce the reaction to foreign tissue is one which I find of the greatest interest

In cases where structure of the graft is the essential thing and a piece of boiled or otherwise killed tissue is quite adequate, why do we have to bother with tissue at all? Is it possible to construct plastic tubes or purely synthetic objects which would do just as well in acting as an

absorbable scaffolding to the host's own tissues? I merely ask the question—perhaps one day it will be answered. The problem of using completely inanimate material for implantation has been approached in the sense that metal tubes and metal plates and so on are used, but what I am suggesting is a substitute for the dead tissue itself which would behave in other respects in the same way as the piece of tissue

That brings us to the second variety of graft, which depends essentially for its value on the functional activity of living cells This opens up the two problems we've heard a great deal about, namely, the acceptability of that tissue to the host, and whether methods of preservation can be evolved which will result in the right material being available just when you want it, obviously a first class essential for practical use In the homografting problem (which was described in some detail and in a most interesting way by Prof Medawar) we can only hope that further information will throw more light on the circumstances in which one can hope for an active homograft to become established and persist, and particularly as to whether anything can be done in the pre-treatment of a graft which will not prevent it developing as a viable graft but may do something to make it more acceptable to the host Some of us have not entirely given up hope that the freeze-drying of tissue without killing it might be possible, and that under particular circumstances viable grafts might be established from freeze-dried tissue If that is possible, it is not inconceivable, and I don't put it higher than that, that the effect of the freeze-drying in decreasing the homograft reaction may be the same as with the dead grafts

The other point about storage is one in which I am particularly interested and therefore I must not say too much about it Ordinary methods of storage do have a very time-limited application, and for the indefinite kind of storage there is no alternative at present for preserving living tissue other than low temperature If low temperature is to be used, then some means must be found of protecting the cell both from the effects of freezing and thawing and from any additional effects there may be of the maintenance at low temperature Just a very few types of cells are apparently able to stand up fairly well to freezing and thawing, but the great majority of mammalian cells do need adventitious protection if they are not to be killed by freezing and thawing and that is where the glycerol story becomes of practical importance Obviously, as Dr Pomerat has emphasized, we are only just at the beginning of that story—every cell we have investigated so far responds quite differently to the addition of glycerol, the treatment in the presence of glycerol and to the subsequent removal of the glycerol, and until those problems, including the rate of freezing and the storage temperatures, can be made to give us more information, we cannot really get any good idea as to the ultimate value of this technique The amount of work to be done which one can see there is quite stupendous I happened to refer to this glycerol matter recently when I was lecturing in the Argentine, and when I'd finished somebody came up who didn't entirely understand what I'd been saying and said "At any rate, Dr.

Parkes you've provided unlimited material for PhD students", and I think that is certainly true

Finally, do we need all these methods for obtaining tissue and preserving it *in vitro* in order to have material for grafting? I think Dr Earle would say, "No, we don't—we're going to make the stuff!" and that perhaps is the ultimate possibility of all this, that tissues will be propagated *in vitro* from generation to generation and year to year and that an appropriate bit will be taken out at the appropriate time for some particular use That's very far in the future, but as a result of this meeting I think we can say that we have some slight glimmer that in the future there may be spare parts for everyone.

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